(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 257 115 A1**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: **86111586.3**

(22) Date of filing: **21.08.86**

(51) Int. Cl.⁴: **C12N 15/00** , C12P 21/02 , C12N 9/30 , C12N 9/34 , C12P 7/06 , C12P 1/00 , C12C 11/04 , C12N 1/16 , A21D 8/04 , C12N 1/18

(43) Date of publication of application:
02.03.88 Bulletin 88/09

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(71) Applicant: **Heineken Technisch Beheer B.V.**
Burg. Smeetsweg 1
NL-2382 PH Zoeterwoude(NL)

(72) Inventor: **Strasser, Alexander**
Zonser Strasse 6
D-4000 Düsseldorf(DE)
Inventor: **Martens, Feodor, Bernard,**
Hogenwoerd 13
NL-2311 HE Leiden(NL)
Inventor: **Dohmen, Jürgen,**
Bretonenstrasse 20
D-4005 Meerbusch 1(DE)
Inventor: **Hollenberg, Cornelius,P.**
Chopinstrasse 7
D-4000 Düsseldorf(DE)

(54) Amylolytic enzyme producing microorganisms, constructed by recombinant DNA technology, and their use in fermentation processes.

(57) This invention provides a method for producing amylolytic enzymes by culturing a microorganism, having received as a result of recombinant DNA technology DNA sequences from a donor yeast comprising the coding sequences for the amylolytic enzymes wherein the host microorgamism is capable of expressing said amylolytic enzymes. Furthermore, this invention provides microorganisms genetically engineered as to being able to produce and express the amylolytic enzymes, a vector containing the DNA sequences, coding for the amylolytic enzymes and the respective DNA sequences. The said host microorganisms are useful in the production of biomass and many fermentation processes, preferably in the production of special beers.

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AMYLOLYTIC ENZYMES PRODUCING MICROORGANISMS, CONSTRUCTED BY RECOMBINANT DNA TECHNOLOGY AND THEIR USE FOR FERMENTATION PROCESSES

Species of the yeast genus Schwanniomyces hydrolyse starch to glucose as the result of the production of two extracellular enzymes, namely amylase (α-1,4-glucan 4-glucanohydrolase E.C.3.2.1.1) and glucoamylase (syn. amyloglucosidase) (α-1,4-glucanglucohydrolase, E.C.3.2.1.3. with debranching activity E.C.3.2.1.9.).

The amylolytic systems of Schwanniomyces castellii and Schwanniomyces alluvius are well documented (Oteng-Gyang et al., 1981; Sills et al., 1982, 1984a, 1984b; Wilson et al., 1982).

It is desirable to work with Schwanniomyces spp. because the α-amylase has the liquefying activity and the glucoamylase has the debranching activity, and because the amylolytic system is sensitive to pasteurisation conditions normally employed in brewing. (Sills et al., 1984a and b).

The yeast Saccharomyces, having none of the mentioned activities of the yeast Schwanniomyces, is the mostly used organism in brewery, baking processes and in the ethanol fermentation industry. In the fermentation processes, starch is conventionally used as a raw material. Since, however, as mentioned, Saccharomyces cerevisiae is not able to hydrolyse starch, the traditional conversion of starch into ethanol and CO_2 by Saccharomyces cerevisiae requires two enzymatic degradation processes prior to the fermentation activity by Saccharomyces cerevisiae, namely liquefication, carried out by adding α-amylase and saccharification by adding glucoamylase with debranching activity (Fogarty et al., 1979).

In the European patent application no. 0 125 615, the use of yeast of the genus Schwanniomyces for the production of low calorie beers has already been described. Using yeast of the genus Schwanniomyces provides an organism in the fermentation procedures which is able to produce the amylolytic enzymes, and therefore the mentioned enzymes do not have to be added prior to the fermentation procedure. Nevertheless, the yeast Schwanniomyces is not at all as efficient and adapted as Saccharomyces cerevisiae in brewery and industrial ethanol production. In the above mentioned European patent application, it has therefore been proposed to use Schwanniomyces in addition to the more efficient genus Saccharomyces, the latter still being the essential microorganism for the fermentation of degraded carbohydrates to ethanol and the starch degrading enzymes from Schwanniomyces. The enzymes are still added separately as usual in the state of the art.

Several amylolytic enzymes encoding genes could be cloned and expressed in Saccharomyces cerevisiae, namely the α-amylase gene of mice (Thomsen, 1983) and of wheat (Rothstein et al., 1984) and the glucoamylase gene of Saccharomyces diastaticus (Yamashita and Fukui, 1983), of Aspergillus niger - (Nunnberg et al., 1984; Innis et al., 1985), Candida albicans (Cohen et al.1985) and Rhizopusoryzae European Patent Application 85 115 910.3).

The amylolytic enzymes produced by the above mentioned donor organisms are, however, not useful for the purposes of the present invention. For the use of genetically manipulated Saccharomyces cerevisiae in brewing and baking procedures and the production of industrial ethanol, it is highly desirable to clone the respective genes derived from very closely related organisms, since the production and application of the expressed enzymes is much more acceptable for the food industry and has the required enzymological and chemical properties for industrial processes. Therefore, the amylolytic enzymes of the microorganism Schwanniomyces, which can be produced by recombinant DNA technology in a number of host microorganisms, are more suitable for a number of applications due to the close relationship of the microorganism Schwanniomyces and the host microorganisms used, and due to the low temperature optimum, the temperature sensitivity, and the high stability of the amylolytic enzymes produced by Schwanniomyces .

The α-amylase gene of Schwanniomyces is the first α-amylase gene cloned from a yeast species and is, as a gene from a narrowly related yeast organism, more compatible with the fermentation processes required in the food industry, for example the brewing and baking processes.

In addition, compared to other α-amylases, the α-amylase of Schwanniomyces has an optimal enzyme activity at a lower temperature of about 37°C, which allows application at lower fermentation or process temperatures. In addition, the α-amylase of Schwanniomyces can be inactivated at 50°C.

The glucoamylase of Schwanniomyces has a special debranching activity and is hence able to hydrolyse starch virtually completely into glucose, whereas the glucoamylase of Candida albicans or Saccharomyces diastaticus has no debranching activity, and hence are not able to hydrolyse starch completely into glucose. The glucoamylase of Schanniomyces, besides its specially suited enzymological

and chemical properties, including optimum of pH and temperature, has the additional advantages over the glucoamylases encoded by other genes already cloned in that this enzyme originates from a yeast species and can be heat inactivated at pasteurisation conditions (Sills et al., 1983) which are normally employed in the brewing process.

It would therefore be most advantageous for the brewing process, the production of industrial ethanol and yeast biomass to provide cultures of Saccharomyces cerevisiae with its high CO_2 production, its good fermentation efficiency, its high ethanol tolerance, with the ability to hydrolyse starch entirely by synthesising and secreting α-amylase and glucoamylase. Also for the CO_2 production in baking, amylolytic enzyme producing strains can be of great advantage.

It was therefore an object of the present invention to provide a microorganism suitable for brewing and baking processes, the production of industrial ethanol and of biomass having the ability to hydrolyse starch entirely if required by being capable of synthesising and secreting α-amylase and glucomaylase.

The desired microorganisms can be provided by genetical manipulation of the microorganisms used by recombinant DNA technology. Recombinant DNA technology is used to clone the commercially interesting genes coding the α-amylase and glucoamylase, whereby DNA fragments are cloned which contain the α-amylase and/or glucoamylase genes of a yeast of the genus Schwanniomyces. After transformation of a host microorganism, the cloned genes coding for amylolytic enzymes are expressed in a host microorganism, for example, Saccharomyces cerevisiae and the gene products are secreted into the medium. The enzymatic pretreatment of starch and/or non-fermentable dextrins, which was necessary to provide the fermenting microorganisms with fermentable sugar , can therefore be avoided, with all advantages with respect to a less expensive and a simplified fermentation processes, for example, in the fields of brewing, production of industrial ethanol and production of biomass. For the baking process, amylolytic yeast can be of great advantage for the preparation of certain types of doughs.

One of the advantages of providing a fermenting microorganism, for example Saccharomyces cerevisiae, having the ability to produce α-amylase and/or glucoamylase is based on the fact that the starch can be continuously and quantitatively degraded, and the biomass or ethanol is simultaneously produced by aerobic or anaerobic con version of the low molecular carbohydrates resulting from the degradation of the starch. In the case of the above mentioned brewing processes, the result of this complete degradation of the starch is a low calorie beer, since the resulting low molecular carbohydrates are easily fermented by the genetically manipulated microorganisms into ethanol and CO_2.

A further example for the use of amylolytic Saccharomyces cerevisiae strains in beer brewing is the production of special beers like low carbohydrate beer, diet beer and special flavoured beer. In addition these strains allow a broadening of the range of starch-containing raw materials for beer brewing.

Although the general idea of the present invention described above comprises the use of various host microorganisms which are suitable in one of the afore mentioned fermentation processes or the production of biomass, a preferred example of constructing a microorganism so as to be able to express and secrete amylolytic enzymes is now described.

For the construction of a yeast of the species Saccharomyces cerevisiae, a transformation system with a particular marker was developed, providing both regulation of the amylase genes and over-production of their gene product in the yeast cells.

According to the present invention, in addition a shuttle cosmid vector has been constructed which can be used as a shuttle vector for transformation of Schwanniomyces, Saccharomyces, Escherichia coli and Schizosaccharomyces pombe.

According to a preferred embodiment of the present invention, the yeast Schwanniomyces castellii acts as a donor for the cloning of α-amylase and/or glucoamylase genes. The yeast Saccharomyces cerevisiae acts as a host yeast being capable of expressing the mentioned genes for the amylolytic enzymes after having received the cloned genes adapted for expression by transformation procedures and further being capable of secreting the gene products.

As a result of genetic manipulation, furthermore, an over-production of the amylolytic enzymes in the yeast Schwanniomyces alluvius is possible. This over-production of amylolytic enzymes in Schwanniomyces strains are of great advantage for the production of these enzymes as such, which after recovering in a known manner, can still be used as described above in fermentation processes in which it is still necessary to add the amylolytic enzymes separately.

The new Schwanniomyces vectors control the function of replication and maintenance in Schwanniomyces. This replication sequence (SwARS1) isolated from Schwanniomyces chromosomal DNA is responsible for autonomous replication in, and high frequency transformation of Schwanniomyces. The SwARS1-element is also functional in Saccharomyces cerevisiae and hence leads to high frequency transformation of Saccharomyces cerevisiae mutants with SwARS1-plasmid.

One suitable representative of Schwanniomyces vectors is pCJD5-1, a hybrid plasmid composed of the SwARS1 sequence, the cos (cohesive) sequence of bacteriophage λ, the pBR322 sequence and the TRP5 gene of Saccharomyces cerevisiae used as a selectable marker.

This plasmid is a new Saccharomyces cerevisiae -Schwanniomyces alluvius -Escherichia coli cosmid shuttle vector with suitable cloning sites for the construction of genomic DNA libraries.

The present invention provides furthermore a new simple and quick transformation procedure for the yeasts Schwanniomyces alluvius, Saccharomyces cerevisiae , Schizosaccharomyces pombe, Kluyveromyces lactis.

Using the above vector and transformation system it is possible to provide new genetically engineered yeast strains of the genus Saccharomyces cerevisiae which are capable of synthesising amylases in culture for mass production.

A consequence of this mass production will be the use of this modified Saccharomyces cerevisiae strain in aerobic and anaerobic starch converting processes to yield yeast biomass, ethanol and CO_2.

According to this invention, new DNA sequences are provided which lead to expression and secretion of proteins in Saccharomyces cerevisiae, Schwanniomyces alluvius, Schizosaccharomyces pombe and Kluyveromyces lactis, since the new DNA sequences contain new secretion signals which allow secretion in the yeasts named.

In addition, sequences are provided by the new DNA sequences which allow regulated gene expression.

Another object of this invention is the provision of a vector without any E. coli sequence for the integration of the amylase genes into the Saccharomyces cerevisiae genome.

Detailed Description of the Present Invention

In the following, a more detailed description of all aspects of the present invention is given.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1:

A: Alignment of plasmid pYc1-αderived by insertion of a 5 kb fragment of S. castelliiDNA, which contains the α-amylase gene, into the cosmid pYc1.

B: Restriction map of the cloned EcoRI fragment of S. castellii .

C: Fragments (1, 2 and 3) subcloned into the plasmid pJDB207 do not lead to functional expression of the α-amylase gene in S. cerevisiae GRF18 transformants.

D: Sequencing strategy:

For sequencing according to Maxam and Gilbert (1980) the EcoRI/SalI fragments were 3' labelled with ^{32}P-dTTP. The orientation and the extension of the sequencing are indicated by arrows.

Figure 2:

The nucleotide sequence of the 5'end of the α-amylase structural gene and its 5' flanking region.

Figure 3:

Amino acid sequences of 5 isolated tryptic peptides of glucoamylase.

Figure 4:

Secreted α-amylase activity of the transformants in different growth phases.

The cells were grown up overnight in precultures with 0.67 % YNB and 4 % glucose. After washing the cells with the medium of the different main cultures, the main cultures were inoculated to O.D.$_{600}$ of 0.2. The main cultures (1 liter YNB medium with the given carbon sources in Fernbach vessels) of the S. alluvius NGA23 transformants were buffered with 0.2 M sodium phosphate buffer pH 6.2 and of the S. cerevisiae MC34 transformants with 0.1 M citrate buffer pH 6.2. The cultures were incubated at 30°C with constant shaking (2 Hertz) on a longitudinal shaker. The measurements of the α-amylase activity and the glucose concentration in the culture supernatant were determined as described. For further details, see specification.

Figure 5:

FPLC ion exchange chromatography on a Mono Q column for separation of the α-amylase protein from concentrated extracellular medium of S. cerevisiae MC34 transformed with pYc1-α.

Using a 10 mM Tris-Cl buffer pH 7.5, the α-amylase could be eluted by an increased NaCl gradient at approximately 300 mM NaCl.

a: A_{254} nm

b: % buffer B
Buffer A: 10 mM Tris-Cl, pH 7.5
Buffer B: 10 mM Tris-Cl, pH 7.5, 2 M NaCl

Figure 6:

Analysis of the purified α-amylase protein from extracellular medium of S. cerevisiae MC34 transformed with the plasmid pYc1-α by SDS-PAGE
Lane a: purified α-amylase
Lane b: molecular weight standard proteins
myosin from rabbit muscle (205 kd); β-galactosidase from E. coli (116 kd); phosphorylase b from rabbit muscle (97 kd); albumin, bovine (66 kd); albumin, egg (45 kd); carbonic anhydrase from bovine erythrocytes (29 kd).

Figure 7:

Thin layer chromatography of starch hydrolysis products.
The α-amylase activity of culture supernatants on soluble starch after 2 hours at 40°C was additionally characterized and determined by a thin layer chromatography of the reaction products using Kieselgel 60 from Merck. The chromatography was done with 2:2:1 acetone - isopropanol - 0.1 M lactic acid solvent system. The chromatogram was developed using naphtoresorcinol as described by Touchstone & Dobbins (1978).

Figure 8:

Construction of S. alluvius plasmids

A a: The plasmid YRp7HIS4 which was isolated from a genomic Sau3A library of S. castellii DNA in the plasmid YRp7 (Struhl et al. 1979) by complementation of the his4-519 mutation of S. cerevisiae AH22, was digested with BamHI and ClaI and religated. One of the resulting plasmids (YpRHIS4) contains a 1.5 kb BamHI/ClaI fragment from the his4 region of S. castellii in YRp7.
b: Deletion of the 1.45 kb ARS1-TRP1-EcoRI fragment from the plasmid YpRHIS4.
c: Insertion of the 5 kb AMY-EcoRI fragment into the plasmid pBRSwARS1.
d: Isolation of the 3.2 kb TRP5-BamHI fragment from the plasmid pYAS1 by preparative gel electrophoresis.
e: Insertion of the 3.2 kb TRP5-BamHI fragment into the plasmids YpRHIS4, pBRSwARS1 and pBRSwARS1-α.

B The plasmids YRp7TRP5-A and -B were constructed by inserting the 3.2 kb TRP5-BamHI fragment from pYAS1 into YRp7. Plasmids with both orientations (-A and -B) of the fragment were obtained.

C The plasmids pEK2TRP5-A and -B were constructed by inserting the 3.2 kb TRP5-BamHI fragment from pYAS1 into the K. lactis vector pEK2 in both possible orientations (-A and -B).

Figure 9:

Copy number estimation of the plasmid YRpJD2 in S. alluvius NGA23 and in S. cerevisiae X3656 2D.
A Ethidium bromide stained plasmid and yeast minilysate DNA separated in a 0.7 % agarose gel.
B Autoradiogram after Southernblot and hybridisation with nick translated YRpJD1 DNA (48 hours exposition at -70°C).
C Autoradiogramm of lanes j-q after 312 hours exposition at -70°C
D Map of the hybridisation probe YRpJD1 (thin line: pBR322; open boxes, S. cerevisiae DNA; black box, S. castellii DNA).
Lanes a-c, control plasmids; a: YRpJD1 EcoRI
b: YRpJD1 uncleaved
c: YRpJD2 uncleaved.
Lanes d-g, yeast minilysate DNA; d-f, DNA of S. alluvius NGA23: YRpJD2 transformants, d and f, cleaved with EcoRI; e and g, uncleaved; h and i, NGA23:YRpJD1 DNA cleaved with EcoRI (h) and uncleaved (i); j and k, DNA of untransformed NGA23 cleaved with EcoRI (j) and uncleaved (k); l and m, DNA of a S. cerevisiae X3656 2D: YRpJD2 transformant cleaved with EcoRI (l) and uncleaved (m); n-q, DNA of untransformed X3656 2D cleaved with EcoRI (n and p) and uncleaved (o and q).

Figure 10:

Copy number estimation of different plasmids in S. alluvius NGA23.
A Ethidium bromide stained EcoRI fragments of control plasmids and yeast minilysate DNA after separation in a 0.7 % agarose gel.
B Autoradiogram after Southern blot and hybridisation with nick translated YRp7HIS4 DNA (15 hours exposition at -70°C).
C Autoradiogram after 6 hours (lanes a-d) and after 125 hours (lanes e-p) exposition at -70°C respectively (faint bands in lanes j and m and the position of the bands of the chromosomal HIS4 locus are indicated by arrows).

D Scheme of the homologies of YRpJD1 and the choromosomal Schwanniomyces HIS4 region to the hybridisation probe YRP7HIS4.
lanes a-e, control plasmids: a, pEK2TRP5-A; b, YRp7TRP5-A, c, pYAS1; d, YRpJD1; lane e, DNA of untransformed S. alluvius NGA23; lane f, DNA of untransformed S. castellii 26076; lanes g-p, DNA of S.alluvius NGA23 transformed with : g, YRpJD1; h and j, YRp7TRP5-A; i, k and l, YRp7TRP5-B; m, pEK2TRP5-A; n,pEK2TRP5-B; o and p, pYAS1 (DNA in lane o is uncleaved).

Figure 11:
Comparison of the copy number of SwARS1 and ARS1 plasmids in S. alluvius and S. cerevisiae.
A Ethidium bromide stained EcoRI fragments of control plasmids and yeast minilysate DNA after separation in a 0.7 % agarose gel.
B Autoradiogram after Southern blot and hybridisation with nick translated YRpJD2-αDNA. The position of the 2.0 kb band of the chromosomal S. cerevisiae TRP5 locus is indicated by an arrow.
lanes a-d, control plasmids: a, pCJD5-1; b, YRpJD2-α; c, YRpJD1; d, YRp7TRP5-A;
lanes e-h, DNA of S. alluvius NJD1; e, f and i, untransformed; g, transformed with pCJD5-1; h, transformed with YRpJD2;
lanes j-m, DNA of S. alluvius NGA23; j, untransformed; transformed with: k, YRpJD2-α; 1, YRpJD2; m, YRpJD1;
lanes n-r, DNA of S. cerevisiae X3656 2D, transformed with: n, YRpJD2-α; o, YRpJD2; p, YRpJD1; q, YRp7TRP5-A; lane r, untransformed.

Figure 12:
In vivo labelling of extracellular proteins from S. alluvius NGA23 transformants.
The transformants were grown under repressed and induced conditions (see Figures 4a, b and c) in 1 ml culture after addition of 100 µCi ^{35}S-methionine (800 Ci/mmol). After incubation at 30°C for different times (18, 22, 28, 43 and 90 hours) 30 µl of the cell-free supernatant were analysed by SDS-PAGE and autoradiography.
a, NGA23:YRpJD2 in YNB, 2 % glucose (repressed);
b, NGA23:YRpJD2 in YNB, 2 % maltose (induced);
c, NGA23:YRpJD2-α in YNB, 2 % glucose (repressed);
d, NGA23:YRpJD2-α in YNB, 2% maltose (induced).
The positions of the molecular weight standard proteins (see Figure 6) are indicated by arrows.

Figure 13:
Restriction map and restriction analysis of the cosmids pCJD5-1 and pCJD5-2
A Restriction map
B Restriction fragments separated in a 0.7 % agarose gel
lane a, lambda DNA cleaved with EcoRI and HindIII;
lane q, lambda DNA cleaved with HindIII;
from left to right:
plasmids YRpJD2, pCJD5-1, pCJD5-2:
lanes b, c, d, cleaved with EcoRI;
lanes h, i, j, cleaved with BamHI;
lanes k, l, m, cleaved with BglII;
lanes n, o, p, cleaved with PvuII.

Figure 14:
Restriction map of the plasmid pKARS2-α.
The 5 kb AMY-EcoRI fragment from plasmid pYc1-α was inserted into the single EcoRI site of the plasmid pKARS2-d.

Figure 15:
A Restriction map of the two subcloned fragments containing the functional glucoamylase gene (AMG).
B The AMG fragments were subcloned into the S. alluvius-S. cerevisiae vector pCJD5-1 resulting in pCJD5-AMG1 and pCJD5-AMG2, into the S.cerevisiae-S.pombe vector pJDB207 resulting in pJDB207-AMG1 and pJDB207-AMG2, and into the K. lactis vector pEK2, resulting in PEK2-AMG2.

Figure 16:
Inactivation of glucoamylase at different temperatures.

Table I

Comparison of preferred codon usage in S. cerevisiae and Schwanniomyces castellii.

amino acid	preferred codons in S. cerevisiae	preferred codons in S. castellii
ala	GCU, GCC	GCU, GCG
ser	UCU, UCC	UCA, UCC, AGC, UCU, AGU (no clear preference)
thr	ACU, ACC	ACU, ACC, ACA
val	GUU, GUC	GUU; GUA, GUC, GUG
ile	AUU, AUC	AUU; AUC, AUA, AAU (no clear preference)
asp	GAC	GAU, GAC
phe	UUC	UUC, UUU (no clear preference)
tyr	UAC	UAU, UAC
cys	UGU	UGU
asn	AAC	AAU, AAC
his	CAC	CAU, CAC
glu	GAA	GAA, GAG
gly	GGU	GGU, GGA
gln	CAA	CAA
lys	AAG	AAA
pro	CCA	CCA, CCU
leu	UUG	UUG, UUA, CUU
arg	AGA	AGA

Table II

Transformation frequencies obtained with the modified method for strains of different yeast genera.

Species	Strain	Plasmid	Replicator	Marker	Transformants per µg DNA
S. alluvius	NGA19	YRpJD1	SwARS1	TRP5	300 - 1000
S. alluvius	NGA23	YRpJD1	SwARS1	TRP5	300 - 1000
S. alluvius	HTR36	YRpJD1	SwARS1	TRP5	300 - 1000
S. cerevisiae	AH22	YRp7HIS4	ARS1	HIS4	500 - 2000
S. cerevisiae	X3656	YRpJD1	ARS1	TRP5	500 - 2000
S. cerevisiae	X3656	pYAS1	2µm	TRP5	1000 - 10000
S. cerevisiae	GRF18	pYc1	2µm	HIS3	200 - 1000
K. lactis	SD11	pEK2	KARS2	TRP1	500 - 1000
H. polymorpha	LR9	pHARS1	HARS1	URA3	500 - 1500
S. pombe	leu1-32	pJDB207	2µm	LEU2	1 - 10

Table III

Transformation frequencies, mitotic stabilities and copy numbers in S. alluvius NGA23

Plasmid	Origin of replication	Trp^+-transformants per µg plasmid DNA	Trp^+-colony forming cells in selective medium	Trp^+-colony forming cells after 15 generations in selective medium	Average copy number per cell	Copy number per Trp^+-colony forming cell
pYe(trp5)1-53	-	0.1-0.2	6 %	0 %	0.1-0.2	2-3
YRp7-TRP5-A	ARS1	5-20	10 %	1 %	0.2-1	2-10
YRp7-TRP5-B	ARS1	5-20	10-20 %	1 %	0.2-1	1-10
pEK2-TRP5-A	ARS1,KARS2	5-20	9 %	1 %	0.2-0.5	2-5
pEK2-TRP5-B	ARS1,KARS2	5-20	11 %	1 %	0.2-0.5	2-5
pYAS1	2µm	50-100	75-90 %	5-10 %	2-3	2-4
YRpJD1	ARS1,SwARS1	300-1000	85-95 %	10-20 %	5-10	6-12

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Table IV

Transformation frequencies, mitotic stabilities and average copy number of the plasmids YRp7-TRp5, YRpJD1 and YRpJD2 in S. alluvius NGA23 and S. cerevisiae X3656.

Plasmid	Origin	Transformants per µg DNA		loss of plasmids after 15 generations in non-selective medium		average copy number per cell	
		S.all. NGA23	S.cer. X3656	S.all. NGA23	S.cer. X3656	S.all. NGA23	S.cer. X3656
YRp7-TRP5-A	ARS1	5-10	500-2000	99 %	80-90 %	0.2-1	5-10
YRpJD1	ARS1,SwARS1	300-1000	500-2000	80-90 %	80-90 %	5-10	5-10
YRpJD2	SwARS1	300-1000	500-2000	80-90 %	80-90 %	5-10	5-10

The yeast strains used are as follows:

Schwanniomyces castellii ATCC 26076.(DSM 3794)
Schwanniomyces alluvius NGA23 and NGS1. (DSM 3792 and 3793)
Kluyveromyces lactis SD11. (DSM 3795)
Schizosaccharomyces pombe leu1-32, his5-303. (DSM 3796)
Saccharomyces cerevisiae GRF18 leu2-3, leu2-112, his3-11, his3-15. (DSM 3797)
Saccharomyces cerevisiae AH22a leu2-3, leu2-112, his4-519, can1. (DSM 3820)
Saccharomyces cerevisiae X3656 2D a, ade, arg4-1, his6, leu1, trp5. (DSM 3798)
Saccharomyces cerevisiae his3 Mal1$^+$ (DSM 3799)

The Escherichia coli strains used are as follows:

Escherichia coli, HB101 F^-, hsd S20, r_B^- m_B^-, recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xy15, mtl-1, supE44, λ^- (DSM 3788)
Escherichia coli JA221, recA-1, leuB6, trpE5, hsdR$^-$, hsdM$^+$, lacY1. (DSM 3789)
Escherichia coli BHB2688, N205 recA$^-$ [imm^{434}, cIts, b2, red^3, Eam4, Sam7]/λ (DSM 3790)
Escherichia coli BHB2690, N205 recA$^-$ [imm^{434}, cIts, b2, red^3m, Dam15, Sam7]/λ (DSM 3791)

The plasmids and cosmids used are as follows:

Cosmid pYC1.
Plasmid pEK2.
Plasmid YRP7.
Plasmid pJDB207.

As far as they are essential for carrying out the present invention, microorganisms as mentioned above have been deposited with the Deutsche Sammlung für Mikroorganismen (DSM).

A complete medium for yeast strains was YEPD (2% bactopeptone, 1% yeast extract, 2% glucose). Minimal medium contained 2% glucose and 0.67% yeast nitrogen base without amino acids (Difco) supplemented with the appropriate amino acids.

Schwanniomyces cells were grown in 2% soluble starch instead of glucose.

Starch fermenting yeast strains were screened for α-amylase and hence halo formation on YNB minimal medium plates containing 1% glucose, 1% soluble starch and 2% agar. The halos were developed by incubating the plates in an iodine saturated chamber for a few seconds.

Bacterial cells were grown in LB medium (0.5% yeast extract, 1% tryptone, 1% NaCl). When required, ampicillin was added to a final concentration of 150 µg per ml, tetracycline to 20 µg per ml, respectively.

Bacterial transformation was carried out as described by R.W. Davis et al. (1980).

Yeast transformations were performed according to the procedure described in this paper.

Throughout this application, various publications are referenced by the name of the author and date of publication within parantheses. Full citations for these references may be found at the end of the specification as an annex, listed in alphabetical order immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Plasmid DNA was purified either by CsCl gradients centrifugation (Maniatis et al., 1982) or by rapid alkaline extraction of plasmid DNA as described by Birnboim and Doly (1979).

Yeast crude extracts were prepared by the method of Schatz (1979).

Yeast minilysates were prepared by the method of Sherman et al.(1983).

DNA fragments were isolated by "low melting agarose" procedure as described by Gafner et al. (1983).

Methods for carrying out the invention

DNA sequencing was carried out as described by Maxam and Gilbert (1980). DNA was nick translated according to Rigby et al. (1977).

Southern hybrisidisation was carried out as described by Southern (1975).

Restriction endonucleases, T4 DNA ligase, E. coli polymerase I, Klenow polymerase can be purchased from Boehringer (Mannheim) and used as specified by the suppliers. α-^{32}P dNTPs and (^{35}S) methionine can be purchased for example from Amersham. Zymolyase 5,000 and zymolyase 60,000 can be obtained from Kirin Brew (Japan). Nitrocellulose filters can be obtained from Schleicher and Schüll.

The cosmid library of Schwanniomyces castellii DNA was constructed in the Saccharomyces cerevisiae shuttle vector pYc1 (Hohn and Hinnen, 1980) and in the Saccharomyces cerevisiae-Schwanniomyces alluvius-E. coli shuttle vector pCJD5-1 constructed according to this invention as described by Ish Horowicz and Burke (1981).

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin used as a standard. The protein was separated by SDS polyacrylamide gel electrophoresis according to the procedure of Laemmli (1970).

Amylase activity was measured kinetically with an enzymatic colour test, for example, α-amylase Mercko test, from Merck. The rate of formation of 2-chloro-4-nitrophenol is determined by photometry at 405 nm at 37°C in 0.1 M potassium phosphate activity in the sample material.

Enzyme units (U) are defined as the amount of enzyme catalysing the formation of 1 µmol 2-chloro-4-nitrophenol x min^{-1} at 37°C.

Glucoamylase activity was measured by a stop assay method: After incubation of the sample in 10% soluble starch in 0.05 M KH_2PO_4-NaOH (pH 5.0) at 50°C. The amount of glucose produced is determined by the glucose dehydrogenase method (system glucose Merck). The quantity of NADH formed is proportional to the glucose concentration.

The enzyme unit (U) is defined as the amount of enzyme catalysing the formation of 1 µmol glucose min^{-1} at 50°C.

Isolated α-amylase from culture supernatant of Schwanniomyces castellii forms a halo on agar plates containing soluble starch after staining with iodine. From these observations, the scheme to clone the α-amylase was based on the assumption that Saccharomyces cerevisiae cells which form no halo can be converted to halo-forming ones when a plasmid vector containing the gene for a starch hydrolysing enzyme, namely α-amylase, is present. To clone the α-amylase gene, a recombinant cosmid library was constructed from genomic DNA of Schwanniomyces castellii ATCC 26076. Using the cosmid cloning

technique described above, a genomic library of about 17,000 independent clones could be generated in the vector pYc1 (Hohn and Hinnen, 1980). The cosmid library was screened for inserts containing the α-amylase gene by transforming Saccharomyces cerevisiae GRF18 to histidine prototrophy and checking for the ability to degrade starch.

Approximately 4500 transformants of GRF18 were screened for their ability to form halos after iodine staining on plates containing leucine, glucose and soluble starch. 13 halo-forming transformants were obtained. From these transformants, total yeast DNA was isolated and used to transform E. coli JA221 cells with selection for ampicillin resistance. Two different recombinant cosmids containing a 32 kb and a 39 kb fragment of Schwanniomyces castellii DNA respectively were obtained. These recombinant cosmids were reintroduced into GRF18. The transformants showed the His^+ and halo-forming phenotype. In a mitotic stability test (Beggs, 1978) both cosmid markers, the HIS3 and the halo-forming function, were segregated out simultaneously, indicating that both genes were present on the cosmid.

The smaller cosmid containing the 32 kb insert was used to identify the DNA region responsible for the halo-forming phenotype. To achieve this, the cosmid was digested with EcoRI, religated and transformed into JA221. The DNA from ampicillin resistant E. coli cells was used to transform GRF18 with selection for histidine prototrophy.

From five halo-forming transformants, total yeast DNA was isolated and used to transform JA221. Plasmid DNA was isolated from ampicillin resistant E. coli transformants. Restriction enzyme analysis revealed that each of these plasmids carries a 5 kb EcoRI Schwanniomyces castellii DNA fragment in the original vector pYc1. This plasmid is called pYc1-α (Fig. 1A). The restriction map of the 5 kb EcoRI fragment is presented in Figure 1B.

Fragments 1, 2 and 3 (Fig. 1C), subcloned into the vector PJDB207 (Beggs, 1981) and transformed into Saccharomyces cerevisiae GRF18 did not result in α-amylase secreting transformants and hence do not contain the functional gene. Therefore, it can be assumed that the functional α-amylase gene spans the HindIII, BglII sites.

Taking into account that the molecular weight of α-amylase is approximately 61,000 daltons (Wilson et al., 1982), it can be predicted that a DNA fragment of approximately 2 kb should contain the functional gene.

To verify this assumption, DNA sequence analysis was performed according to Maxam and Gilbert (1980). For sequencing, both EcoRI-SalI fragments of the 5 kb EcoRI fragments were radioactively labelled at the SalI site (Fig. 1D). DNA sequence analysis revealed a 5' noncoding region followed by an open reading frame spanning the SalI site. The DNA sequence is presented in Fig. 2.

The TATA box (Gannon et al., 1979) usually located 25-32 bp upstream and the CAAT box (Benoist et al., 1980) which has been found about 80 bp upstream from the transcription initiation point, are thought to be important for transcription initiation. While the TATA box is present in almost all examined genes, the CAAT box is not always present.

In the regulation region of the α-amylase gene there is a perfect TATA box at -89 and a CAAT box at -115.

In the 5' region of the α-amylase gene, there is a CAAG sequence at position -7. The codon usage of the α-amylase is given in Table I.

Expression of the Schwanniomyces α-amylase gene and secretion of the protein by Saccharomyces cerevisiae

GRF18:pYc1-α transformants are not able to grown on soluble starch as a sole carbon source. This could be shown to be due to a defect in maltose utilisation. Therefore, the plasmid pYC1-α was used to transform Saccharomyces cerevisiae MC34 (his3), having a Mal^+ phenotype with selection for starch degradation. All histidine phototroph transformants were able to grow on starch as a sole carbon source and could form halos surrounding the colony after staining with iodine. For α-amylase activity measurements, MC34:pYc1-α transformants were cultivated with selection for histidine prototrophy in YNB medium containing glucose.

During the growth of Saccharomyces cerevisiae in unbuffered YNB medium, the pH drops to approximately 3.5.

The α-amylase becomes inactivated below a pH of 4 and has a pH optimum at about 6.2 (Sills and Stewart, 1984). Therefore, the medium was buffered with 0.1 M citrate buffer pH 6.2.

During growth of MC34:pYc1-α, crude extract and extracellular medium was assayed for α-amylase activity.

α-amylase activity could only be detected in the extracellular culture medium and not in crude extracts, indicating that the α-amylase is secreted. The total activity in the extracellular medium decreases at late logarithmic growth (Fig. 4D). This loss of enzymatic activity could be explained by proteolytic degradation of α-amylase, because proteolytic activities accumulate during the transition from the logarithmic to stationary growth phase (Ferguson et al., 1973). However the stability of α-amylase could not be improved by adding the protease inhibitor PMSF. It can therefore only be concluded that the inactivation is not due to a serin protease.

The total α-amylase activity in the extracellular medium of stationary grown MC34:pYC1-α cells in complete medium YEPD was significantly higher, compared to cells grown in YNB medium. The most likely explanation is the two-fold higher proteolytic activities when Saccharomyces cerevisiae is grown inYNB, compared to growth in YEPD (Tsai et al., 1973).

Sometimes enzymes can be stabilized by the addition of substrate (Katsunuma et al., 1972). MC34:pYc1-α transformants which are grown in YNB containing soluble starch as a sole carbon source showed no difference in the α-amylase activity profile during different growth phases, compared to cells grown in glucose as a sole carbon source, indicating that the α-amylase could not be stabilised in the presence of substrate.

However, it was observed that MC34:pYc1-α had an initial lag period in its ability to ferment starch either by an induction period at maltose uptake or probably due to a time dependent accumulation of secreted α-amylase into the medium.

From the recent investigations, it could be shown that the α-amylase gene is also expressed and the gene product secreted by Schizosaccharomyces pombe and Kluyveromyces lactis. For this purpose, Schizosaccharomyces pombe was transformed with plasmid pYclα (Fig. 1) and Kluyveromyces lactis with plasmid pKARS2-α (Fig. 14a). The former transformants could form halos on starch plates after iodine staining. Quantification of the α-amylase system by these two transformed yeast strains is the subect of the present investigation.

Moreover, the α-amylase gene was cloned into a wild type bottom-fermenting brewers' yeast strain of Saccharomyces cerevisiae var. carlsbergensis by transformation with the plasmid containing α-amylase gene (pYC1-α) and screening for α-amylase activity by halo formation on plates containing soluble starch.

Isolation and characterisation of the enzymes α -amylase and glucoamylase from Schwanniomyces castellii

Both enzymes were isolated from the medium of a continuous culture of Schwanniomyces castellii.

Medium: YNB + 1% starch; 20 mM PO_4; pH 6.3.

Conditions: temp. = 30°C; PO2 = 70 %; D = 0.15 h^{-1}.

Yeast cells were separated from the medium by ultrafiltration (UF) and the filtrate was concentrated with an UF-membrane, cut-off 10,000 D. The concentrated filtrate was loaded on a DEAE-Sephacel column in 20 mM phosphate buffer, pH 5.6.

Glucoamylase was eluted from the column between 150-180 mM NaCl, α -amylase was eluted between 180-250 mM NaCl. Fractions containing enzyme activity were collected, dialysed against 100 mM Ammonium acetate buffer pH 6.5 and concentrated by Amicon YM 10 filtration.

Aliquots of ± 4 mg of each protein were further purified using HPLC gel permeation chromatography on a TSK 2000 SW column using 100 mM ammonium acetate buffer pH 6.5.

The enzymes purified in this way were tested for homogeniety by SDS-PAGE, resulting in one single protein band for each enzyme.

Preparation of glucoamylase peptide fractions

Glucoamylase (50 mg) was incubated with trypsine (1 mg) for 24 hr., 37°C in 50 mM ammonium acetate buffer pH 6.5, resulting in ± 30% breakdown of the enzyme. Peptides were separated by means of HPLC on a TSK 2000 SW column and further purified on RPHPLC using C18 and C8 columns. Elution was carried out with a gradient system 0-100% acetonitrile. From 5 peptides, the amino acid sequence was determined (see Fig. 3).

Characterisation of glucoamylase

MW: 120,000 D
pH optimum 5.0
temp.optimum 50°C
Km: 1.0 - 1.5 % dextrin (Serva)
pI 3.5
glycosilated
activity repressed by glucose
temp. inactivation - see Fig. 16.

Characterisation of α-amylase

MW 53,000 D
pH optimum 6.0
temp. optimum 37°C
Km: 0.004 - 0.07 % soluble starch

Isolation of secreted α-amylase from Saccharomyces cerevisiae

The maximal measurable secreted α-amylase activity from MC34:pYc1-transformants growing in YNB medium could be obtained from cells of the early logarithmic phase (Fig. 4D). It is advantageous to isolate the α-amylase from extracellular YNB medium instead of complex medium containing yeast extract and bactopeptone, because the initial specific activity (α-amylase activity/protein ratio) is significantly higher in YNB than in YEPD.

In addition, since the amount of secreted α-amylase is directly correlated to the amount of actively growing yeast cells, a stepwise growth of MC34:pYc1-α in YNB (selection on the plasmid), YEPD (optimal growth condition), and finally YNB (secretion of α-amylase from a high amount of actively growing cells) was used for the isolation of secreted α-amylase. In detail, the transformants were grown in YNB medium containing glucose. When an optical density of approximately 4 at 600 nm was reached (late logarithmic growth phase), the cells were harvested by centrifugation and resuspended in prewarmed (30°C) YEPD to a final OD_{600} of 7.5.

After 2 hours growth at 30°C, the cells were sedimented and resuspended in the same volume of prewarmed (30°C) YNB containing glucose and 0.1M citrate buffer pH 6.2. After 4 hours incubation at 30°C (optical density at 600 nm reached a value of 17), the α-amylase was purified from concentrated extracellular medium by means of FPLC ion exchange chromatography using a Mono Q column (Pharmacia). The α-amylase protein could be eluted in 10 mM Tris-Cl, pH 7.5 at 300 mM NaCl using an increasing NaCl gradient (Fig. 5).

The active protein fraction was concentrated by lyophilisation and analyzed by SDS polyacryiamide gel electrophoresis (Fig. 6). The presence of only one band with a molecular weight of about 55,000 D showed that the α-amylase protein was highly purified by this procedure.

For enzyme characterisation, the products of starch degradation by the isolated α-amylase were analysed by thin layer chromatography (Fig. 7). The products of starch hydrolysis are maltose and large amounts of maltotriose and higher oligosaccharides. Glucose is not produced in detectable amounts. This is in agreement with the above mentioned observation, that GRF18(Mal^-):pYc1-α transformants are not able to grow on starch as a sole carbon source.

Transformation of Schwanniomyces alluvius

One of the prerequisites for the establishment of a transformation system is the availability of an appropriate mutant which can be functionally complemented by a cloned gene.

Various tryptophan auxotrophic Schwanniomyces alluvius mutants were analysed for their defect in the L-tryptophan biosynthetic pathway. The mutants were tested for having a defect in the last step of L-tryptophan biosynthesis, namely the condensation reaction of indole and L-serin, which in Saccharomyces cerevisiae is catalysed by tryptophan synthetase.

A defect in this last step leads to an accumulation of indole (Manney et al., 1969). The tryptophan auxotrophic mutant NGA23 accumulates indole when grown in YNB medium. In addition, compared to other tryptophan auxotrophs, NGA23 cannot grow on YNB containing indole. It was therefore concluded that NGA23 has a mutation in the tryptophan synthetase gene. NGA23 exhibits a low reversion rate (10^{-8}) and is thus suitable for complementation studies with the cloned Saccharomyces cerevisiae TRP5 gene (Carbon et al., 1977) coding for tryptophan synthetase gene (Walz et al., 1978; Zalkin et al., 1982). The initial intention was to transform NGA23 via integration.

The transformation experiment was based on the assumption that there is no extensive homology between the TRP5 gene of Saccharomyces cerevisiae and the mutated tryptophan synthetase gene of Schwanniomyces alluvius as revealed by Southern hybridisation of nick translated pYAS1 (Strasser, 1982) DNA to chromosomal DNA of Schwanniomyces alluvius.

From a partial Sau3A bank of Schwanniomyces DNA in YRP7, a 9 kb DNA fragment was cloned, resulting in plasmid YRP7HIS4 complementing the his4 mutation of Saccharomyces cerevisiae strain AH22 (Fig. 8).

It was assumed that a 1.5 kb BamHI-ClaI fragment from this HIS4 region of Schwanniomyces castellii should have enough homology to direct integration into the genome of Schwanniomyces alluvius NGA23 via homologous recombination. Therefore, the plasmid YRpJD2 was constructed (Fig. 8), containing the Saccharomyces cerevisiae Trp5 gene as a selectable marker and the 1.5 kb BamHI-ClaI fragment.

YRpJD2 was used to transform NGA23 by the protoplast method described by Beggs (1978) screening for tryptophan prototrophy on YNB plates. Five transformants per µg plasmid DNA were obtained.

It seemed unlikely that this surprisingly high transformation frequency was due to integration events, taking into account that the plasmid was not linearised prior to transformation, which would have increased the probability for integration (Orr-Weaver et al., 1981) and that the regeneration frequency of Schwanniomyces using the protoplast method was lower than 0.1%.

The transformation of NGA23 with YRpJD2 is based on autonomous replication as was indicated by the instability of the Trp^+ phenotype after growth of the transformants under non-selective conditions in YEPD. After 15 genertions, 80-90 % of the cells had lost the ability to grow on selective medium.

The autonomous replication was ascertained by transforming E.coli cells with yeast minilysates and retransformation of Schwanniomyces NGA23. In addition, Southern analysis showed the presence of original YRpJD2 sequences (Fig. 9) and thus exclude integration of the TRP5 gene as a cause of the tryptophan prototrophy.

No transformants except for some abortive transformants could be obtained after transformation of NGA23 with pYe(trp5)1-53 (Walz et al., 1978),which does not contain the 1.5 kb fragment of Schwanniomyces castellii indicating that this Schwanniomyces DNA fragment contains a sequence which allows autonomous replication of plasmid YRpJD2 in Schwanniomyces alluvius NGa23.

Therefore, it is concluded that the 1.5 kb DNA fragment of Schwanniomyces castellii in the vector YRpJD2 contains an autonomous replication sequence, namely SwARS1.

Transformation procedure

The regeneration of Schwanniomyces cells using the protoplast method (Beggs, 1978) for transformation is approximately 0.1% Because the regeneration rate or protoplast formation correlates directly with the transformation frequency, attempts were made to change the conditions for protoplast formation using different concentrations of helicase or zymolyase and different incubation times for the transformation of YRpJD2 into NGA23.

Using helicase, no transformants could be obtained. Only by using zymolyase (1 mg/ml) for 30 min at 37°C with a regeneration rate of 0.1 %, 5 transformants per µg YRpJD2 plasmid DNA could be obtained. Shorter incubation resulted in an increase of the regeneration frequency to approximately 0.5%, but no transformants were obtained.

These findings demonstrate that the protoplast formation (and not the regeneration frequency and hence the enzymatic degradation of the cell wall) is the critical step in the transformation of Schwanniomyces using the protoplast method. Therefore, the transformation procedures described by Ito et al. (1983) and Klebe et al. (1983) were tested.

Both procedures do not require the enzymatic degradation of the cell wall. With the lithium sulphate method of Ito et al. (1983), no transformants were obtained. However, using the freeze-thaw method of Klebe et al. (1983) NGA23 could successfully be transformed with YRpJD2 plasmid, yielding reproducibly 50-100 transformants per µg YRpJD2 plasmid DNA.

Using the method of Klebe et al (1983), log phase yeast (10 ml) was washed with 5 ml of SBEG (1 M sorbitol, 10 mM bicine, pH 8.35, 3% ethylene glycol) by centrifugation at 1000 x g for 3 min at 22°C. All subsequent incubations were carried out at 30°C. The pellet was resuspended in 0.2 ml SBEG; and after 5 min, 1 to 20 µl plasmid DNA was added. After 10 min, the preparation was placed in a -70°C freezer for 10 min (or longer) and thawed by rapid agitation in a 37°C water bath. 1.5 ml of 40% purified PEG 1000 (Klebe et al., 1983), 200 mM bicine pH 8.35 (stored frozen) was added; and after a 1 h incubation, the cells were washed with 2 ml 0.15 mM NaCl, 10 mM bicine pH 8.35. After centrifugation, the pellet was resuspended in 1 ml 0.15 mM NaCl, 10 mM bicine pH 8.35, and plated on selective medium. Using the conditions described above, the plasmid DNA is added before the cells are frozen at -70°C. However, the possibility of adding the DNA to the frozen cells just before thawing would be of advantage to have competent cells at any time for the transformation with any plasmid available.

The attempts of adding YRpJD2 DNA to frozen cells of the Schwanniomyces alluvius mutant NGA23 were successful in that the same transformation frequencies (50-100 transformances per µg) as compared to the original procedure described above were obtained.

Klebe et al. (1983) describes that the PEG used is critical for reproducible transformation. Therefore Klebe et al.(1983) performed a time consuming PEG 1000 preparation for optimal transformation efficiency.

To circumvent this tedious procedure, the influence of several PEGs from Serva and Roth company was tested. Until now, the optimal transformation efficiencies were obtained using PEG 1000 from Roth company. Substitution of 1 M sorbitol in SBEG by 1.25 M KCl, 30 mM $CaCl_2$ leads to an increase in the transformation frequency by a factor 2, yielding 200 NGA23 transformants per µg YRpJD2 plasmid DNA.

During freezing of yeast cells, the plasma membrane becomes damaged, resulting in a higher permeability; in addition, the degree of cell damage is dependent on the velocity of freezing (Lepock et al., 1984). Based on these observations, the transformation competence of the cells was tested after freezing at -70°C in a freezer (relatively slow freezing), with dry ice acetone at -78°C (fast freezing) and with liquid nitrogen -196°C (very fast freezing).

Using liquid nitrogen, the transformation frequency decreased by 40%, whereas the usage of dry ice acetone increased the yield of transformants by 30% compared to freezing in a -70°C freezer.

The DNA was added to the frozen cells (stored at -70°C for 10 min up to 6 months) and thawed by rapid agitation at 37°C. Instead of rapid agitation in a 37°C water bath, an Eppendorf mixer, type 5432, at 37°C was used. This condition for rapid agitation during thawing gave the best reproducible transformation frequencies.

With these modifications, a transformation frequency of 300-1000 transformants per µg YRpJD2 plasmi DNA was obtained for Schwanniomyces alluvius NGA23 mutants.

With this simplified transformation procedure, high transformation frequencies could also be obtained for Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Kluyveromyces lactis (Table II).

Estimation of plasmid copy number in Schwanniomyces alluvius and Saccharomyces cerevisiae transformants

With our simplified transformation procedure, Schwanniomyces alluvius NGA23 was transformed with:
pYe(trp5)1-53 - containing pBR313 (Bolivar et al., 1977) and the TRP5 gene of Saccharomyces cerevisiae
pYAS1 - a 2 µM derivative containing both the LEU2 and TRP5 genes of Saccharomyces cerevisiae (Strasser, 1982)
YRP7TRP5 - containing ARS1 and both the TRP1 and TRP5 genes of Saccharomyces cervisiae
YRpJD1 - containing both SwARS1 and ARS1 and both the TRP1 and TRP5 genes of Saccharomyces cerevisiae
YRpJD2 - containing SwARS1 and the TRP5 gene of Saccharomyces cerevisiae
pEK2TRP5 - containing both KARS1 and ARS1 and the TRP5 gene of Saccharomyces cerevisiae

The plasmids are summarised in Figure 8.

The transformation frequencies and stabilities of these plasmids in Schwanniomyces alluvius NGA23 are summarised in Table III. The copy numbers of the different plasmids were estimated by Southern blot analysis (Fig. 9 and 10).

The chromosomal HIS4 locus representing one copy per cell was used as an internal standard. There are three chromosomal ÉcoRI fragments (0.9 kb, 6.0 kb, 8.0 kb) which hybridize with ^{32}P-labelled YRP7HIS4 DNA. The homology of the chromosomal HIS4 locus and of the YRpJD1 plasmid to the hybridisation probe YRP7HIS4 is represented in Figure 10D.

For copy number estimation of the plasmids pYAS1, YRP7Trp5, YRpJD1, (YRpJD2) and pEK2TRP5, the intensity of the common 5.3 kb EcoRI band was compared with the 6 kb EcoRI band of the chromosomal HIS4 gene.

The intensity of staining in the autoradiogram reveals that the plasmids YRpJD1 and YRpJD2 have a copy number of about 5-10 copies per cell (Fig. 9 and 10).

Both the plasmids YRP7TRP5 and pEK2TRP5 have about the same average copy number per cell, which is in the range of about 0.2-1, independent of the orientation of the TRP5 fragment, whereas pYAS1 exists in about 2-3 copies per cell (Fig. 10).

ARS1 and 2 µm DNA sequences of Saccharomyces cerevisiae can be used for autonomous replication in Schwanniomyces alluvius NGA23 although not as efficiently as SwARS1. SwARS1 behaves in Schwanniomyces alluvius as ARS1 in Saccharomyces cerevisiae, yielding 5-10 copies per cell (Fig. 11, Table IV). The plasmid YRpJD1 which has the ARS1 element in addition to SwARS1 element, showed no increased copy number as compared to YRpJD2, which only has the SwARS1 element. The plasmid pEK2TRP5, which has the KARS2 element in addition to the ARS1 element, has the same copy number as the plasmid YRp7TRP5, which only has the ARS1 element. It is therefore assumed that KARS2 cannot serve as an efficient autonomous replication element in Schwanniomyces alluvius.

The transformation of Schwanniomyces alluvius NGA23 with pYe(trp5)1-53 (Walz et al., 1978) can be explained by an abortive transformation event concerning the high instability of these transformants (Table III).

Regulation of the clone Schwanniomyces castellii α-amylase gene and over-production of the protein in Schwanniomyces alluvius transformants

The production of α-amylase in the yeast species Schwanniomyces underlies catabolite repression in the presence of glucose (Sills et al., 1982).

The synthesis of α-amylase is induced by soluble starch dextrins and maltose (Sills et al., 1982). Maltose, which is part of the products after starch degradation, was found to be a stronger inducer than soluble starch (Sills et al., 1984b). It appears that a very low basal level of amylase is constitutively produced. This low level of α-amylase can hydrolyse starch, releasing low molecular weight sugar such as maltose, which can penetrate the cell membrane and in turn induce the synthesis of α-amylase (Sills et al., 1984b).

To study regulation and expression of the cloned α-amylase gene in Schwanniomyces alluvius, the 5 kb EcoRI fragment containing the structural α-amylase gene was inserted into the YRpJD2 (Fig. 8), resulting in plasmid YRpJD2-α and transformed into Schwanniomyces alluvius NGA23 with selection for tryptophan prototrophy on YNB plates.

As it is shown in Figure 5B, the total α-amylase activity of NGA23: YRpJD2-α transformants growing under induced condition in YNB maltose medium is increased 4-5 fold compared to control transformants NGA23: YRpJD2 which do not contain additional plasmid encoded α-amylase (Fig. 4A).

During the induction phase of α-amylase synthesis, the drastical 7-fold increase in extracellular α-amylase activity per cell concentration (OD_{600nm}) compared to control transformants, correlates with the estimated 5-10 copies of SwARS1 plasmids in Schwanniomyces alluvius.

It is therefore concluded that the increase in α-amylase activity is due to a gene dosage effect.

When the NGA23:YRpJD1 and NGA23:YRpJD2 transformants were grown under repressed conditions in YNB containing 2% glucose, no α-amylase activity could be measured in the extracellular medium until the glucose concentration dropped to about 0.2 mM (Fig. 4C and E). Thus the plasmid encoded α-amylase is regulated by catabolite repression as chromosomal α-amylase.

By construction of the α-amylase structural gene under control of a high expression promoter as e.g. that of PDC1 or PGK genes, higher expression strains independent of growth stage and C-source can be obtained.

The overproduction of the α-amylase protein was shown by analysis of in vitro labelled proteins of the culture supernatant by fluorography (Fig. 12). α-amylase activity could be measured in the extracellular medium of MC34:pYC1α transformants under growth in YNB containing 2% and also 4% glucose as a sole carbon source indicating that the plasmid encoded α-amylase is not regulated by catabolite repression in Saccharomyces cerevisiae.

Isolation of auxotrophic mutants of Schwanniomyces alluvius

For prosecuting the cloning of the glucoamylase gene, the functional complementation of a glucoamylase mutant of Schwanniomyces alluvius was chosen.

A prerequisite is the availability of an appropriate mutant which can be complemented by the cloned gene. For this purpose, a Schwanniomyces alluvius mutant NGS1 which is not able to grow on maltose or soluble starch sole carbon source was analysed. When this mutant was grown on plates containing 1% glucose and 1% soluble starch, halo formation could be observed after iodine staining, indicating that α-amylase can be secreted by the mutant. No glucoamylase activity could be detected in crude extracts and culture supernatant.

As already mentioned, α-amylase can hydrolyse starch into dextrin, maltose and higher oligosaccharides (Fig. 7), whereas glucoamylase can use these products as substrates for the enzymatic hydrolysation into glucose. The interesting feature of the NGS1 mutant is the inability to grow on maltose. The Schwanniomyces alluvius wild type secretes glucoamylase which hydrolyses maltose to glucose in the extracellular medium, and hence, in contra to Saccharomyces cerevisiae, has no need for a maltose uptake system. It is therefore concluded that NGS1 is an α-amylase$^+$ glucoamylase$^-$ mutant, in having no functional glucoamylase either by a point mutation in the structural gene, or in the promoter region.

We have focused our attention on this glucoamylase mutant to clone the glucoamylase gene of Schwanniomyces castellii by functional complementation.

For cloning purposes, an additional mutation had to be introduced into the α-amylase$^+$ glucoamylase$^-$ mutant, preferentially a trp5 mutation (leading to a defect in the indole tryptophan reaction), because Schwanniomyces alluvius could be efficiently transformed using the TRP5 gene of Saccharomyces cerevisiae as a selectable marker on SwARS1 containing plasmids e.g. YRpJD1 and YRpJD2. In addition, these mutants can easily be identified by their property of indole accumulation.

For the isolation of trp5 auxotrophic mutants, all procedures were carried out at 30°C. 10 ml NGS1 culture was grown to OD_{600}= 0.6 in complex medium, washed twice with H_2O and exposed to UV light (254 nm) for 50 seconds (1% survivals). The mutagenised cells were then incubated for 2 days on complex medium for segregation of the trp auxotrophs and plated on YEPD plates for replica plating on minimal medium containing tryptophan and on minimal medium plates containing all amino acids but no tryptophan.

From 2614 NGS1 colonies tested, 7 trp auxotrophic mutants which grow on minimal medium containing tryptophan but not on minimal medium containing all amino acids except tryptophan could be isolated (reversion rate 10^{-7} -10^{-8}). Two of these seven mutants could be analysed to be defective in the indole tryptophan reaction since both were accumulating indole.

These trp5, α-amylase$^+$glucose$^-$ mutants were designated NJD1 and NJD2. These two strains could be efficiently transformed with YRpJD1 yielding approximately 800 transformants per µg DNA,indicating that NJD1 and NJD2 are really mutated in the TRP5 gene.

Further cloning procedures can be carried out in an analogous manner as with the α-amylase gene.

Construction of an E. coli - Saccharomyces cerevisiae - Schwanniomyces alluvius cosmid shuttle vector for cioning genes in Schwanniomyces alluvius

In analogy the the cosmid pYc1 (2µ) and pYc2 (ARS) which were constructed for cloning genes in Saccharomyces cerevisiae (Hohn and Hinnen, 1980), a cosmid for cloning in Schwanniomyces alluvius was constructed.

Cosmids are plasmids which contain the cos (cohesive sequence) region of bacteriophage lambda (Collins and Hohn, 1978) as well as the essential sequences for cloning (replication origins, a gene as selectable marker and suitable cloning sites). The cos sequence comprises the structural information necesary for packaging the DNA to produce an intact bacteriophage lambda (Hohn, 1975). High molecular oligomeric cosmid DNA can therefore be efficiently packaged by an in vitro packaging system (Hohn, 1979), if the cos sequences are separated by any 37-52 kb DNA fragments corresponding to about 73% to 105% of the lambda phage genome (Feiss et al., 1977). The relatively large size of clonable fragments presents the possibility that only about 1,200 cosmid clones containing fragments with an average size of about 30-40 kb are necessary to encompass the genome of Saccharomyces cerevisiae by more than 95% (Hohn and Hinnen, 1980).

For cloning large DNA fragments, the advantage in using cosmid instead of plasmid DNA is that relatively high molecular oligomeric DNA can easily be obtained by ligation of highly concentrated DNA, whereas the efficiency to form circular transformable plasmids by ligation of large DNA fragments with smaller vector DNA is disadvantageous because of the relation between the fragment size and effective concentration of free ends (Dugaiczyk et al., 1975). In addition, the packaged DNA can efficiently be transduced by infection of lambda sensitive E. coli cells (Hohn and Hinnen, 1980).

Based on these observations, an E. coli -Schwanniomyces alluvius -Saccharomyces cerevisiae cosmid shuttle vector was constructed by subcloning the 1.7 kb BglII fragment of plasmid pHC79 containing the cos region (Hohn and Collins, 1981) into one of the two BamHI sites of YRpJD2. YRpJD2 was therefore partially digested with BamHI (0.5 units/µg DNA at 30°C) and the resulting largest linear DNA which arises from a single cleavage by BamHI was isolated by preparative gel electrophoresis and ligated with the 1.7 kb BglII fragment of pHC79, which has also been isolated by preparative gel electrophoresis after complete digestion of pHC79 DNA. This ligation mixture was used to transform E. coli JA221, selecting on ampicillin resistance. Subclones were screened by colony hybridisation using the nick translated 1.7 kb BglII fragment as a radioactive probe.

From the positive clones,two different cosmids, namely pCJD5-1 and pCJD5-2 (Fig. 13a) could be isolated and analysed after cleavage with EcoRI, BamHI and PvuII restriction enzymes (Fig. 13b). Figure 13b demonstrates that in cosmid pCJD5-1, the cos sequence is subcloned into the BamHI site of YRpJD2 between the SwARS1 sequence and the TRP5 gene. In pCJD5-2, the cos region was inserted in tandem at the same position. The existence of two, in tandem, orientated cos elements should drastically increase the packaging efficiency compared to cosmids which contain only a single cos element (Lindenmeier et al., 1982). Up till now, it was not tested whether this is also the case of pCJD5-2.

With cosmids pCJD5-1 and pCJD5-2, 500-1000 NJD1 transformants per µg cosmids DNA were obtained by selecting on tryptophan, indicating that the cos element does not influence the transformation frequency. Both cosmids contain

(a) the SwARS1 sequence which acts as replication origin in Schwanniomyces alluvius as well as Saccharomyces cerevisiae

(b) the origin for replication of E. coli;

(c) the ampicillin resistance gene as selectable marker in E. coli ;

(d) the TRP5 gene of Saccharomyces cerevisiae as selectable marker in Schwanniomyces alluvius as well as in Saccharomyces cerevisiae trp5 mutants; and

(e) the cos region as prerequisite for the construction of a cosmid library.

In addition, both cosmids contain suitable cloning sites, namely BamHI and SalI for cloning Sau3A, BamHI, BglII, SalI and XHOI fragments, and hence provide a new class of cosmid vectors for the construction of a genomic DNA library to clone genes in E. coli , Saccharomyces cerevisiae and Schwanniomyces alluvius.

Cloning of a DNA fragment of Schwanniomyces castellii which functionally complements the glucoamylase mutation of NJD1

The cloning of the glucoamylase gene was started from a Schwanniomyces castellii ATCC 26076 cosmid pool which was established by ligating partially BamHI digested genomic DNA of Schwanniomyces castellii into the E. coli - Saccharomyces cerevisiae - Schwanniomyces alluvius shuttle vector pCJD5-1 using the cosmid cloning technique described by Ish-Horowicz et al., 1981.

After in vitro packaging of the DNA ligation mixture, E. coli strain HB101 was infected, yielding approximately 15,000 ampicillin resistant colonies corresponding to approximately 30 genome size equivalents of Schwanniomyces castellii assuming the same genome size for Schwanniomyces castellii as for Saccharomyces cerevisiae (approx. 15,000 kb) and a cloned DNA fragment ranging between 30 and 40 kb in size. From these transductants, cosmid DNA was isolated and used for the transformation of NJD1 (α-amylase$^+$glucoamylase$^-$trp5)with selection for trp prototrophy on minimal medium containing 2% glucose.

The transformation frequencies using both the protoplast method (Beggs, 1978) and the simplified freeze thaw method was relatively low (1-10 transformants/µg cosmid DNA). Nevertheless approx. 3,000 transformants could be obtained which were screened for their regained ability to grow on soluble starch as sole carbon source. One NJD1 transformant which has a Trp$^+$ and glucoamylase$^+$ phenotype could be isolated from starch plates. In a mitotic stability test (Beggs, 1978), both the TRP5 phenotype and the glucoamylase complementing functions were segregated out, simultaneously indicating that both genes were carried by the cosmid. The cosmid was rescued by transforming E. coli HB101 with yeast minilysate

and selection for ampicillin resistance. Cosmid DNA was isolated and used for retransformation of NJD1, to ascertain that the glucoamylase complementing function is still carried by the isolated cosmid DNA. After cleavage of the cosmid with BamH1, two BamH1 sites could be localised within the cloned fragment, dividing it into the three fragments, two of them being of approx. 10 kb and 12 kb.

In order to decrease the size of the approx. 40 kb size cosmid, BamHI fragments were subcloned into the single BamHI of pCJD5-1 plasmid. Therefore, the cosmid DNA was digested with BamHI, religated and transformed into E. coli HB101. The plasmid DNA from ampicillin resistant cells was used to transform NJD1 and to screen for growth on soluble starch as a sole carbon source. Plasmid DNA was rescued by transforming HB101 with yeast minilysate and selection for ampicillin resistance. Plasmid DNA was isolated for restriction analysis after digestion with BamHI endonuclease.

Figure 15 demonstrates that the glucoamylase complementing function is carried by the 12 kb BamHI fragment, subcloned into pCJD5-1. This plasmid is designated pCJD5 -AMG1 (Fig. 14). Since this plasmid contains the SwARS1 element acting in Saccharomyces cerevisiae as well as inSchwanniomyces alluvius, the cloned gene in Saccharomyces cerevisiae was analysed by transforming X3656 selecting on tryptophan prototrophy. Unfortunately the transformants were not able to use soluble starch as a sole carbon source. The first explanation, that the 5-10 copies of pCJD5-AMG1 in Saccharomyces cerevisiae are not high enough to overcome possible proteolytic degradation, failed, since after subcloning of the approx. 12 kb BamHI fragment into the high copy number, plasmid pJDB207 (Beggs, 1981) (Fig. 14) having 50-100 copies per cell, and transformation of GRF18 Saccharomyces cerevisiae cells, none of the transformants were able to grow on soluble starch as a sole carbon source.

Although glucoamylase activity could be measured in NJD1:pCJD5-AMG2 transformants, no activity in both crude extract and culture filtrate of Sacharomyces cerevisiae transformants could be detected. Because Käufer et al. (1985) have recently shown that Schizosaccharomyces pombe has a considerable advantage over Saccharomyces cerevisiae in accurately excising an intervening sequence from a transcript of a higher eucaryotic gene, attempts were made to express the glucoamylase in this fission yeast. However, no expression and secretion of glucoamylase could be detected in Schizosaccharomyces pombe leu1-32 (complemented with LEU2 of Saccharomyces cerevisiae after transformation with plasmid pJDB20^{7} -AMG^{1} (Fig. 14). The transformation of the gene into Kluyveromyces lactis SD11(trp1) with pEK2-AMG2 (Fig. 14)did not lead to transformants which can grow on soluble starch as sole carbon source. It is therefore concluded that a Schwanniomyces gene was cloned which cannot be functionally expressed in Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Kluyveromyces lactis because of different splicing specificites, promoter recognition or Schwanniomyces specific post transcriptional processing.

For further analysis, subcloning smaller fragments of the 12 kb DNA was carried out in order to find the smallest possible DNA fragment still carrying the functional complementation property. The molecular weight of glucoamylase is approx. 117,000 d (Simoes-Mendes, 1984) corresponding to a long structural gene of about 3 kb in length.

Furthermore, the gene can be sequenced to analyse codon usage and possibly existing introns. Additionally, the 5' non-coding leader sequence of Schwanniomyces castelliiDNA can be exchanged by a well characterised promoter such as the ADH1 (Hitzeman et al., 1981) or PDC promoter (Hollenberg et al., 1983) to exclude possible inefficiency of the Schwanniomyces promoter in other yeast species.

In order to characterise the glucoamylase gene, glucoamylase was isolated for the production of glucoamylase specific antibodies to assay the crude extracts and culture filtrate of NJD1 as well as Saccharomyces cerevisiae or Schizosaccharomyces pombe and Kluyveromyces lactis transformants for the existence of inactive glucoamylase. In addition, peptides of purified glucoamylase were isolated for amino acid sequencing in order to synthesise synthetic oligonucleotides for analysing the cloned gene.

Genetically manipulated microorganisms, preferably yeasts, as described above in this specification are of great advantage in a lot of fermenting processes because of their ability to hydrolyse higher molecular carbohydrates.

One of the possible uses of the genetically altered yeast strains described above is in the production of biomasses. Since the yeast strains having the new ability to secrete α-amylase and glucoamylase still remained there other good fermentation abilities biomasses can be produced most efficiently by the use of the inventive yeast strains. The methods of producing the biomasses are the usual ones, known by the man skilled in the art.

A further field of use of Saccharomyces cerevisiae, having the new ability of producing α-amylase and glucoamylase is the production of bakers yeast and of bread, using the bakers yeast.

The inventive yeast can be used as pellet yeast, for example, made by convential drum-drying processes or can be powdered yeast, for example, having a particle size of 1.7 mm or less. Making the powdered yeast comprises spray-drying of a liquid yeast composition in air, followed by further drying, as known in the state of the art. The inventive yeast, for example, may be prepared as a dried yeast as described in GB-PS 1 459 407, GB-PS 1 459 085 or GB-PS 1 230 205. Any other methods known in the art for producing bakers yeast or producing baked products by using the new yeast of this invention are also suitable.

The genetically manipulated yeast strains provided according to this invention are further highly suitable for the production of ethanol. The preferred organism for the production of ethanol by fermentation is the yeast Saccharomyces cerevisiae and the preferred carbohydrate used in the ethanol production is a start. Thus, a Saccharomyces cerevisiae, being able to ferment starch quantitively is highly advantageous in the production of ethanol. The production of potable spirit or industrial ethanol by the use of the genetically manipulated yeast strain of the present invention can be carried out in a manner known per se. The inventive yeast strain has the ability to ferment concentrated carbohydrate solutions, has ethanol tolerance and the ability to produce elevated concentrations of ethanol, has a high cell viability for repeated recycling and has temperature tolerance.

Possibly the most important field of use of the new Saccharomyces cerevisiae, being able to produce α-amylase and glucoamylase is the production of special beers, preferrably low carbohydrate beers. As already described in the specification above, one technique for producing low carbohydrate beers was to add amylolytic enzymes to the wort during fermentation. Because 70% to 75% of the dextrins in wort are of the branched type, debranching enzyme is essential for total hydrolyses of wort dextrins to fermentable sugars. The glucoamylase, produced by the new genetically manipulated Saccharomyces cerevisiae possesses debranching activity and therefore can hydrolyse the dextrins completely. An important characteristic of the amylolytic enzymes produced by the new yeast Saccharomyces cerevisiae is their sensitivity to the normal pasteurisation cycle employed in brewing.

The method of preparing beers has a series of stages, starting with malting, where barley is soaked in water and allowed to germinate and during this step amylase is released which converts the starch from the grain into sugar. During the next step, grains are removed by filtration and the resulting liquid wort is brewed with hops. The hops give the beer the bitter flavour and during this step the enzymatic conversion of starch to sugar is stopped. Next, the wort is cooled and filtered to remove the precipitated proteins. Then yeast is added to the wort to convert the sugers to alcohol and carbon dioxide in a fermentation process, after which the mixture is stored, the yeast is settling out, the beer is filtered again, bottled or canned and pasteurised.

In order to produce a low-calorie beer, it is desirable to consume as many of the carbohydrates as possible in the fermentation process. For this purpose the new Saccharomyces cerevisiae, being able to produce α-amylase and glucoamylase is highly suitable.

A method for preparing the low-calorie beer can be carried out generally as described in the European Patent Application 0 163 135, with the exception that no amylolytic enzymes have to be added during the brewing process. The above-described usual brewing steps can be carried out without any time-consuming or expensive additions of amylolytic enzymes. Carrying out the brewing process as known in the art, using the new genetically manipulated Saccharomyces cerevisiae according to this invention, results in a special beer, preferably low-carbohydrate beer, low-calorie beer depending on the degree of the fermentation of sugar. The brewing process, using the Saccharomyces cerevisiae of this invention is decreased in time due to a more rapid decrease in Es at the end of the fermentation process.

Using the Saccharomyces cerevisiae of this invention, the manufacturing process can be adapted for the production of a beer from a high gravity wort, the carbohydrate level of which can be regulated by a predetermined fermentation time and dilution thereafter. Furthermore, the manufacturing process may be adapted for the fermention of substrates, the constituent of which are for a considerable part suspended hydrated carbohydrates. The genetically engineered Saccharomyces cerevisiae can be brought into contact with the fermented wort subsequently to main fermentation but prior to conditioning.

A more detailed description of the production of a low-calorie beer is now given in the following examples.

Example I

Beer brewing using a Saccharomyces cerevisiae genetically manipulated as described above

Brewers' wort was prepared using conventional brewing techniques such that 80% of the extract in the wort was derived from brewers' malt and 20% derived from corn grits. This wort had an original gravity of 12° Plato. 2.5 liter fleakers were filled with 2 liters wort and pitched with 1.5 g/l wet yeast of a genetically manipulated Saccharomyces cerevisiae . Fermentation was allowed to proceed at a controlled temperature of 9°C under anaerobic conditions and with mechanical stirring due to the small volume of fermentation. At the end of fermentation, the beer was cooled to 0°C, yeast was separated from the beer and analyzed for residual extract and % ethanol. Also a typical gas chromatogram of the young beer was made to determined the amount of esters and higher alcohols.

Example II

The fermentation process was the same as for Example I except that the fermentation temperature was 15°C. The fermentation was faster and the resulting beer had a better aroma, higher glucose and higher alcohol content.

Example III

The fermentation process was the same as Example II, except that the prepared wort had an original gravity of 15° Plato.

Example IV

Partially purified starch derived from wheat, maize or rice was suspended in an appropriate buffer, boiled in order to gelatinaze the starch and liquefied by treatment with a commercial available α-amylase enzyme preparation.

This solution was fermented with a genetically manipulated Saccharomyces cerevisiae at a temperature of 30°C.

After fermentation had ceased completely, the ethanol concentration was measured.

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Claims

1. A method for producing polypeptides, preferably amylolytic enzymes, by culturing a host microorganism under suitable conditions and recovering of said polypeptides in a manner known per se, **characterised in that** the host microorganism, as a result of recombinant DNA technology, has received DNA sequences from a donor yeast comprising the coding sequences for said polypeptides and is capable of expressing said polypeptides.

2. A method according to claim 1, **characterised** in that the said polypeptides are α-amylase and/or glucoamylase.

3. A method according to one of the claims 1 or 2, **characterised** in that the host microorganism is selected from a group consisting of yeasts of the genera Saccharomyces, Schizosaccharomyces, Schwanniomyces or Kluyveromyces, or bacteria of the genus Zymomonas or moulds of the genus Aspergillus.

4. A method according to claim 3, **characterised** in that the yeast is Saccharomyces cerevisiae.

5. A method according to one of the claims 1 to 4 **characterised** in that the donor yeast is selected from a group consisting of the genera Saccharomycopsis, Lipomyces, Pichia, Schwanniomyces, Debaryomyces or Saccharomyces.

6. A method according to claim 5 **characterised** in that the donor yeast is Schwanniomyces castellii ATCC26076.

7. A method according to one of the claims 1 to 6, **characterised** in that the transfer of the DNA sequences from the donor yeast into the host microorganism is carried out by transformation.

8. A method according to claim 7, **characterised** in that the transformation is carried out by a vector, preferably a DNA fragment, a plasmid or a cosmid.

9. A method according to claim 8, **characterised** in that the vector contains homologous DNA, preferably derived from Schwanniomyces, leading to integration into the host genome.

10. A method according to one of the claims 8 or 9 **characterised** in that the vector contains one or more DNA sequences which control the function of replication and maintenance within the Schwanniomyces cells, preferably the autonomously replicating DNA sequences ARS, most preferably the DNA sequences SwARS originating from Schwanniomyces.

11. A method according to one of the claims 8 to 10 **characterised** in that the vector is pCJD5-AMG1 and/or YRpJD2-α.

12. A method according to one of the claims 7 to 11, **characterised** in that the host yeast cells before the transformation procedure are subjected to fast freezing, which preferably is carried out with dry ice acetone at temperatures of about -70° to -80°C.

13. A method according to one of the claims 7 to 12 **characterised** in that the transformation is carried out during thawing and rapid agitation of the frozen yeast cells.

14. A method according to one of the aforegoing claims **characterised** in that the host yeast is specifically glycosylating the secreted polypeptide.

15. A method according to one of the aforegoing claims **characterised** in that the production of α-amylase is used as marker in Kluyveromyces lactis, Saccharomyces cerevisiae and Schizosaccharomyces pombe for transformation under non-selective conditions.

16. An α-amylase produced according to the method of one of the claims 1 to 15.

17. An α-amylase according to claim 16, **characterised** in that it has its temperature optimum at about 37°C and is inactivated at about 60°C.

18. An α-amylase according to claims 16 or 17, **characterised** in that it is encoded by the DNA sequence depicted in Figure 2 and all modifications of said DNA sequence which retain the function of the encoded α-amylase.

19. A glucoamylase produced according to the method of one of the claims 1 to 15.

20. A glucoamylase according to claim 19 **characterised** in that it has its temperature optimum at about 50°C, and is inactivated at about 60°C and is capable of hydrolysing barley starch completely into glucose.

21. A microorganism, preferably useful as a host microorganism in a method according to one of the claims 1 to 15, **characterised** in that it is capable of expressing an α-amylase according to claims 16 to 18 and/or a glucoamylase according to claims 19 or 20 as a result of having received DNA sequences from a donor yeast comprising the coding sequences for said α-amylase and/or glucoamylase by recombinant DNA technology.

22. A microorganism according to claim 21, **characterised** in that it is selected from a group consisting of yeasts of the genera Saccharomyces, Schizosaccharomyces, Schwanniomyces, or Kluyveromyces or bacteria of the genus Zymomonas or moulds of the genus Aspergillus.

23. A yeast cell according to claim 22, **characterised** in that it is Saccharomyces cerevisiae.

24. A yeast cell according to claim 22 **characterised** in that it is of the genus Schwanniomyces, preferably the species Schwanniomyces alluvius.

25. A yeast cell according to claim 23 **characterised** in that it is useful in brewing and baking processes, the food industry, and fermentation processes.

26. A yeast cell according to claim 23 **characterised in** that it is useful for complete fermentation of starch into ethanol.

27. A yeast cell according to claim 23 **characterised** in that it is preferably useful in producing special beer by being capable of fermenting dextrins.

28. A vector carrying foreignDNA sequences which are coding for a polypeptide, **characterised** in that said polypeptides are amylolytic enzymes, preferably α-amylase and/or glucoamylase, wherein the vector is preferably useful in a process of one of the claims 1 to 15.

29. A vector according to claim 28, **characterised** in that it can be used as a shuttle vector for E-scherichia coli -Saccharomyces cerevisae -Schwanniomyces alluvius -Schizosaccharomyces pombe.

30. A vector according to claims 28 or 29, **characterised** in that the vector is pCJD5-AMG1 or YRpJD2-α.

31. A vector according to claims 28 or 29, **characterised** in that it is selected from a group consisting of the plasmids YRpJD2, pCJD5-1 or pCJD5-2.

32. A DNA sequence **characterised** in that it is coding for a polypeptide, preferably an amylolytic enzyme, most preferably an α-amylase and/or glucoamylase, and is capable of expressing said polypeptide in a suitable host microorganism, preferably a microorganism according to one of the claims 21 to 27 and is preferably useful in a method according to one of the claims 1 to 15.

33. A DNA sequence according to claim 32 **characterised** in that it has a sequence depicted in Figure 2.

34. A DNA sequence according to claims 32 or 33, **characterised** in that it is coding for an α-amylase according to one of the claims 16 to 18, and is obtained by recombinant DNA technology from natural and/or cDNA and/or chemically synthesised DNA.

35. A DNA sequence according to claim 34, **characterised** in that it comprises the DNA sequence as depicted in Figure 2, encoding the α-amylase, the amino acid sequence of which is depicted in Figure 2.

36. A DNA sequence **characterised** in that it is coding for a glucoamylase according to claims 19 or 20 and is obtained by recombinant DNA technology from natural and/or cDNA and/or chemically synthesised DNA.

37. A combination of DNA sequences **characterised** in that it comprises a structural gene encoding an amylolytic enzyme and one or more DNA sequences capable of regulation of the expression of the structural gene in a microorganism, preferably, a microorganism according to one of the claims 21 to 27.

38. A combination of DNA sequences according to claim 37, **characterised**in that it comprises a structural gene encoding an amylolytic enzyme of a yeast.

39. A combination of DNA sequences according to claim 38, **characterised** in that the structural gene is encoding amylolytic enzymes of the genus Schwanniomyces, preferably of the species Schwanniomyces castellii.

40. A combination of DNA sequences according to one of the claims 37 to 39, **characterised** in that said structural gene, coding for an amylolytic enzyme is encoding an α-amylase.

41. A combination of DNA sequences according to claim 40 **characterised** in that the structural gene has the DNA sequence depicted in Figure 2, and is encoding a polypeptide having the amino acid sequence which is depicted in Figure 2.

42. A combination of DNA sequences according to one of the claims 37 to 39 **characterised** in that the structural gene coding for an amylolytic enzyme encodes a glucoamylase.

43. A combination of DNA sequences according to one of the claims 37 to 42 **characterised** in that the DNA sequence comprises all modifications of the DNA sequences retaining their coding function for an amylolytic enzyme.

44. A combination of DNA sequences according to one of the claims 37 to 43 **characterised** in that the structural gene contains DNA sequences derived from the structural gene coding for α-amylase or glucoamylase which modify said protein product while retaining its functions in such a way that said protein product is secreted into the cell wall or the culture medium.

45. A combination of DNA sequences according to claim 44 **characterised** in that it is contained in a host microorganism according to one of the claims 21 to 27, said host microorganism being suitable in a process for preparing a polypeptide according to one of the claims 1 to 15.

46. A beer manufacturing process **characterised** in that a yeast is used which has received by recombinant DNA technology DNA sequences from a donor yeast which encodes amylolytic enzymes, preferably α-amylase and/or glucoamylase, wherein the yeast used is preferably Saccharomyces cerevisiae.

47. A beer manufacturing process according to claim 46 **characterised** in that a yeast being capable of expressing an α-amylase and/or a yeast being capable of expressing glucoamylase of a yeast being capable of expressing α-amylase and glucoamylase is used.

48. A process according to claims 46 or 47 **characterised** in that the manufacturing process is adapted for the production of a special beer, preferably low carbohydrate beer, low calorie beer or diet beer.

49. A process according to one of the claims 46 to 48, **characterised** in that the manufacturing process is adapted for the production of beers containing levels of aroma components comparable to beers obtained by fermentation of high glucose worts.

50. A process according to one of the claims 46 to 49 **characterised** in that the process is decreased in time due to a more rapid decrease in apparent extract (Extrakt scheinbar = Es) at the end of the fermentation process.

51. A process according to one of the claims 46 to 50 **characterised** in that the manufacturing process is adapted for the production of a beer from a high gravity wort, the carbohydrate level of which can be regulated by a predetermined fermentation time and dilution thereafter.

52. A process according to one of the claims 46 to 51 **characterised** in that the manufacturing process is adapted for the fermentation of substrates, the constituents of which are for a considerable part suspended hydrated carbohydrates.

53. A process according to one of the claims 46 to 52 **characterised** in that the genetically engineered yeast is brought into contact with the fermented wort subsequently to main fermentation, but prior to conditioning.

54. An ethanol manufacturing process **characterised** in that a yeast is used which has received by recombinant DNA technology DNA sequences from a donor yeast which encodes amylolytic enzymes, preferably α-amylase and/or glucoamylase, wherein the yeast used is preferably Saccharomyces cerevisiae.

55. A fermentation process **characterised** in that a yeast is used which has received by recombinant-DNA technology DNA sequences from a donor yeast which encodes amylolytic enzymes, preferably α-amylase and/or glucoamylase, wherein the yeast used is preferably Saccharomyces cerevisiae according to claim 23 and wherein said fermentation process is adapted for the production of beverages produced from fermented or distilled fermented substrates containing suspended hydrated starch.

56. A process for the production of baker's yeast **characterised** in that a yeast is used which has received by recombinantDNA technology DNA sequences from a donor yeast which encodes amylolytic enzymes, preferably α -amylase and/or glucoamylase, wherein the yeast used is preferably Saccharomyces cerevisiae according to claim 23.

57. A process of preparing bread in a manner known per se, **characterised** in that a yeast according to claim 56 is used.

58. A process for the production of biomass **characterised** in that a yeast is used which has received by recombinant DNA technology DNA sequences from a donor yeast which encodes amylolytic enzymes, preferably α-amylase and/or glucoamylase, wherein the yeast used is preferably Saccharomyces cerevisiae according to claim 23.

59. A transformation system for the transfer of DNA sequences from a donor microorganism to a host microorganism, **characterised** in that the host microorganism is subjected to fast freezing before adding the DNA, preferably that the fast freezing is carried out with dry ice acetone at temperatures of about -70°C to -80°C.

FIG. 1

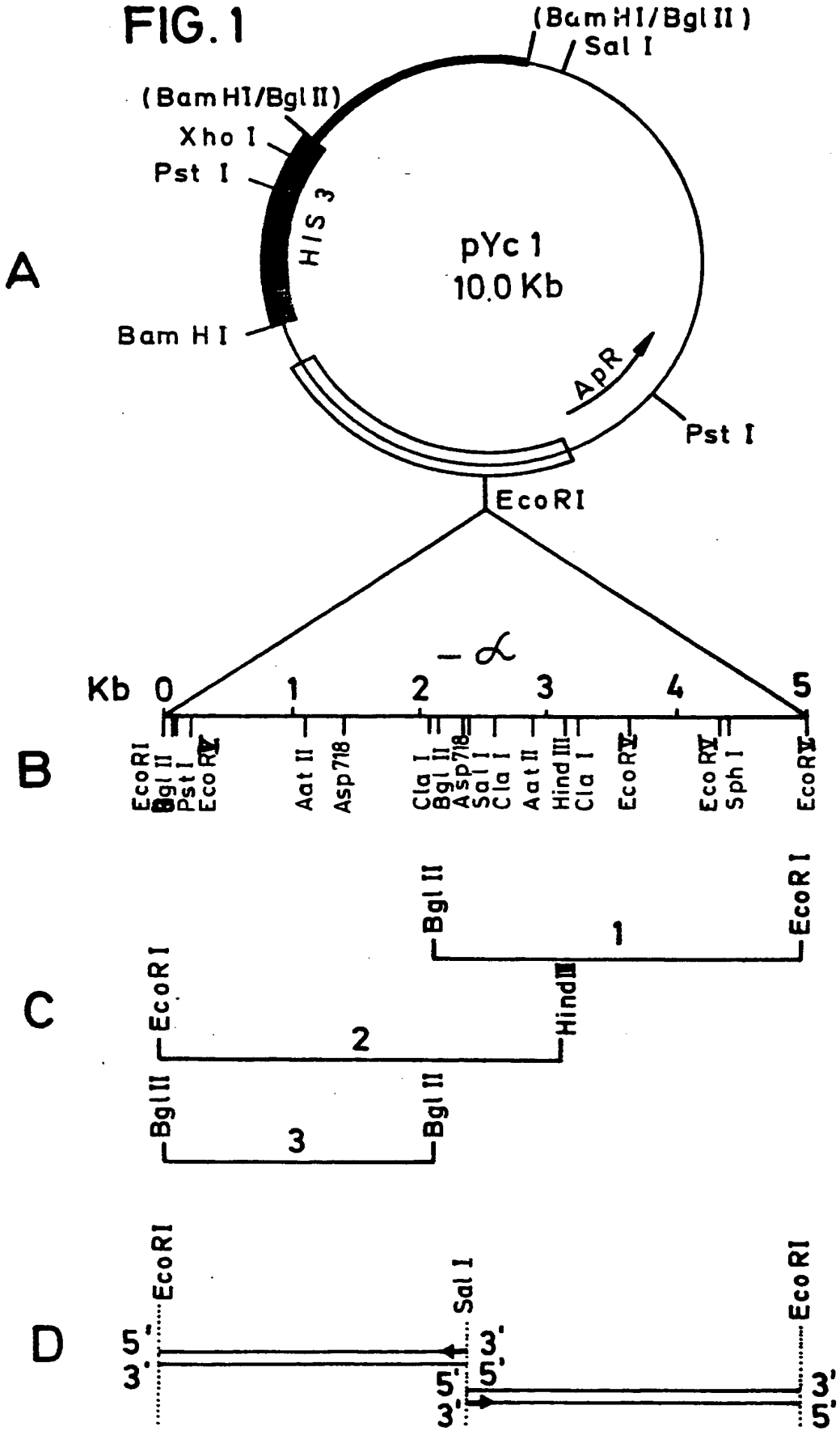

A
(Bam HI/Bgl II)
Sal I
(Bam HI/Bgl II)
Xho I
Pst I
HIS 3
pYc 1
10.0 Kb
Bam H I
ApR
Pst I
EcoRI
B
Kb 0 1 2 3 4 5
EcoRI
Bgl II
Pst I
EcoRV
Aat II
Asp718
Cla I
Bgl II
Asp718
Sal I
Cla I
Aat II
Hind III
Cla I
EcoRV
EcoRV
Sph I
EcoRV
C
Bgl II
EcoR I
1
EcoR I
Hind III
2
Bgl II
Bgl II
3
D
EcoRI
Sal I
EcoRI
5'
3'
3'
5'
5'
3'
3'
5'

Fig2 (1)

```
        10         20         30         40         50         60
GGTACCTGAGCTAAATTTAGAACCGGCTATAGATCCGCTTGTCTAAAGAAGAGATAATGA

        70         80         90        100        110        120
AGAAAACAATTAACCGAGCACTCTTATTAAGTTTTTTTCTATTTTCTTTTGCTCCTACTT

       130        140        150        160        170        180
CAATAATTTATCTAAATTGTATTGTGCGTTAGATCAGAATGTACTGATAACAGAGAGTAT

       190        200        210        220        230        240
TATCATACACTTGTGGATTTCAAAAGGCGGAATCAAAAGCATACGTAGTCAAACCCTTGG

       250        260        270        280        290        300
TTATTTGATGCAATTAAGGTTGTAGTCGTTCTTACCGATCCATCATTATACCCCACACGG

       310        320        330        340        350        360
TTTCATGGTATGTAGGTGTTTCAATAGTGAAGTACAATGAATGTTTTGGTAATGCTGTAT

       370        380        390        400        410        420
GTGGATCAGTAATTATGTTAAACAATTAAGTCTGAAAATTTATTAAAATTTTACCTACAA

       430        440        450        460        470        480
ATTAAGCCGAAATCCAATCGAAGGTGCCGCCCAGCTGGTGTATAAATTACTCTTGAAATT

       490        500        510        520        530        540
CAAGTTGAACGTTGATCTCTCTAAAGCAAAGCTGTTATTCTACAATACTAAATAAAATAA

       550        560        570        580        590        600
AAGCAAGACATGAGATTTTCAACTGAAGGATTTACAAGTAAAGTTGTTGCAGCAATTTTA
         MetArgPheSerThrGluGlyPheThrSerLysValValAlaAlaIleLeu

       610        620        630        640        650        660
GCATTCTCAAGATTGGTATCTGCTCAACCGATTATTTTTGACATGAGAGATGTTAGCTCG
AlaPheSerArgLeuValSerAlaGlnProIleIlePheAspMetArgAspValSerSer

       670        680        690        700        710        720
TCAGCTGATAAATGGAAAGACCAATCGATTTATCAAATCGTTACTGATAGGTTTGCCAGA
SerAlaAspLysTrpLysAspGlnSerIleTyrGlnIleValThrAspArgPheAlaArg

       730        740        750        760        770        780
TCTGATGGCTCGACCACAGCTGACTGTTTAGTGAGTGATCGCAAGTACTGTGGTGGATCT
SerAspGlySerThrThrAlaAspCysLeuValSerAspArgLysTyrCysGlyGlySer

       790        800        810        820        830        840
TATAAAGGGATTATCGACAAGTTGGATTATATTCAAGGTATGGGTTTCACTGCGATCTGG
TyrLysGlyIleIleAspLysLeuAspTyrIleGlnGlyMetGlyPheThrAlaIleTrp

       850        860        870        880        890        900
ATCTCCCCAGTTGTTGAGCAAATTCCTGACAATACTGCTTATGGTTATGCTTACCATGGT
IleSerProValValGluGlnIleProAspAsnThrAlaTyrGlyTyrAlaTyrHisGly
```

Fig.2 (2)

```
      910        920        930        940        950        960
TATTGGATGAAAAATATTGATGAATTGAACACTAATTTTGGTACCGCTGATGAATTGAAA
TyrTrpMetLysAsnIleAspGluLeuAsnThrAsnPheGlyThrAlaAspGluLeuLys

      970        980        990       1000       1010       1020
CAATTAGCTAGCGAATTGCATTTCAGAAGCATGTATGATGGTCGACGATGTTACAACCAT
GlnLeuAlaSerGluLeuHisPheArgSerMetTyrAspGlyArgArgCysTyrAsnHis

     1030       1040       1050       1060       1070       1080
TATGCTTGGAACGGAGATGGTTCAAGCGTAGATTATTCTAGTTTCACTCCATTCAATCAA
TyrAlaTrpAsnGlyAspGlySerSerValAspTyrSerSerPheThrProPheAsnGln

     1090       1100       1110       1120       1130       1140
CAATCTTACTTCCACGATTATTGTTTGATTACAAATTATAATGATCAAACCAATGTTGAA
GlnSerTyrPheHisAspTyrCysLeuIleThrAsnTyrAsnAspGlnThrAsnValGlu

     1150       1160       1170       1180       1190       1200
GATTGTTGGGAAGGTGATACTGAAGTCTCCCTTCCAGATTTAAGTACCGAGGATAATGAA
AspCysTrpGluGlyAspThrGluValSerLeuProAspLeuSerThrGluAspAsnGlu

     1210       1220       1230       1240       1250       1260
GTTATAGGAGTATTTCAAACTTGGGTGTCAGATTTTGTTCAAAACTATTCAATCGATGGT
ValIleGlyValPheGlnThrTrpValSerAspPheValGlnAsnTyrSerIleAspGly

     1270       1280       1290       1300       1310       1320
TTAAGAATTGATAGTGCAAAGCACGTAGATACCGCTTCATTAACGAAGTTTGAGGACGCT
LeuArgIleAspSerAlaLysHisValAspThrAlaSerLeuThrLysPheGluAspAla

     1330       1340       1350       1360       1370       1380
TCTGGTGTTTATAACTTAGGTGAAGTTTATCAAGGAGATCCAACTTATACTTGTCCATAT
SerGlyValTyrAsnLeuGlyGluValTyrGlnGlyAspProThrTyrThrCysProTyr

     1390       1400       1410       1420       1430       1440
CAGAATTATATGAAAGGAGTTACCAACTATCCATTATACTATCCAGTATATAGATTCTTC
GlnAsnTyrMetLysGlyValThrAsnTyrProLeuTyrTyrProValTyrArgPhePhe

     1450       1460       1470       1480       1490       1500
AGTGATACTTCGGCGACTTCCAGTGAGTTAACTTCAATGATCTCCACGTTACAGTCATCT
SerAspThrSerAlaThrSerSerGluLeuThrSerMetIleSerThrLeuGlnSerSer

     1510       1520       1530       1540       1550       1560
TGTTCGGACGTCTCTTTGTTGGGAAACTTTATTGAAAACCATGACCAAGTTAGATTTCCA
CysSerAspValSerLeuLeuGlyAsnPheIleGluAsnHisAspGlnValArgPhePro

     1570       1580       1590       1600       1610       1620
TCAGTTACCTCAGACACATCCTTGATTAAGAATGACATGGCTTTTATAATTTTGGGTGAT
SerValThrSerAspThrSerLeuIleLysAsnAspMetAlaPheIleIleLeuGlyAsp

     1630       1640       1650       1660       1670       1680
GGTATCCCAATTATTTATTATGGCCAAGAACAAGGTCTCAATGGTGGTTCCGATCCTGCC
GlyIleProIleIleTyrTyrGlyGlnGluGlnGlyLeuAsnGlyGlySerAspProAla

     1690       1700       1710       1720       1730       1740
AATAGAGAAGCTTTATGGTTAAGTGGATATAATACCGATTCAGAATACTACGAGCTAATC
AsnArgGluAlaLeuTrpLeuSerGlyTyrAsnThrAspSerGluTyrTyrGluLeuIle

     1750       1760       1770       1780       1790       1800
AGTAAACTAAATCAAATAAGAAATCAAGCTATTAAGAAGGATTCTGCCTATTCAACTTAC
SerLysLeuAsnGlnIleArgAsnGlnAlaIleLysLysAspSerAlaTyrSerThrTyr
```

```
      1810       1820       1830       1840       1850       1860
AAATCCTCAGTTGTTTCTTCTTCAGACCACTATATAGCCACTAGGAAGGGTAGCGATGCT
LysSerSerValValSerSerSerAspHisTyrIleAlaThrArgLysGlySerAspAla

      1870       1880       1890       1900       1910       1920
AATCAATTGATTTCCATTTTTAATAATTTAGGTTCAAACGGGTCACAGGATATTACTGTC
AsnGlnLeuIleSerIlePheAsnAsnLeuGlySerAsnGlySerGlnAspIleThrVal

      1930       1940       1950       1960       1970       1980
AGCAACACCGGCTATTCTAGTGGTGATAAAGTTATCGATATTATTTCTTGCAATTCCGTT
SerAsnThrGlyTyrSerSerGlyAspLysValIleAspIleIleSerCysAsnSerVal

      1990       2000       2010       2020       2030       2040
TTAGCTGGTGACTCCGGAAGCTTATCTGTATCAATTTCTGGTGGAATGCCACAAGTTTAC
LeuAlaGlyAspSerGlySerLeuSerValSerIleSerGlyGlyMetProGlnValTyr

      2050       2060       2070       2080       2090       2100
GCTCCGTCCTCTGTTCTTTCGGGATCTGGCATCTGCAATCAATAGATTGATCCAGCGCTA
AlaProSerSerValLeuSerGlySerGlyIleCysAsnGlnEnd

      2110       2120       2130       2140       2150       2160
ACCCTTTTTTTAGCAACGACAAGTTTATTTTAGAAAAAGTTTTCTAAGAATGGTCAAAAC

      2170       2180       2190       2200       2210       2220
AAGTTCTTATTACTTCTATGTCCCTGGATATCTGTTTTCAATGTTCTCTGACTCCACATT

      2230       2240       2250       2260       2270       2280
CCTCATGTTTAGTTCTCTATTTTTTGTCGATCTTCTAAGTTTTTTTATTCTTAATTTTAA

      2290
TCCAAAAGTT
```

Fig.3

Peptide 1: GLY-GLY-ASN-VAL-LEU-PRO-THR-GLU-GLN-PRO-GLY-TYR-THR-VAL-ALA-GLN-SER-ARG

Peptide 2: ALA-THR-SER-TYR-PRO-VAL-GLY-PHE-ASN-VAL-SER-CYS-ALA-SER-GLN-TRP-LYS

Peptide 3: GLY-VAL-PHE-PRO-GLY-ALA-GLY-LYS-GLU-VAL-TYR-TYR-ASP-TRP-TYR-THR-GLN-ARG

Peptide 4: TRP-GLY-TYR-ASP-THR-ILE-GLN-LYS

Peptide 5: THR-LEU-PHE-ALA-ASN-ASP-VAL-GLY-ASP-PRO-ILE-ASP-GLY-ASN-ILE-TYR-GLY-VAL-HIS-PRO-VAL-TYR-LEU-ASP-GLN-ARG

Fig.4 (1)

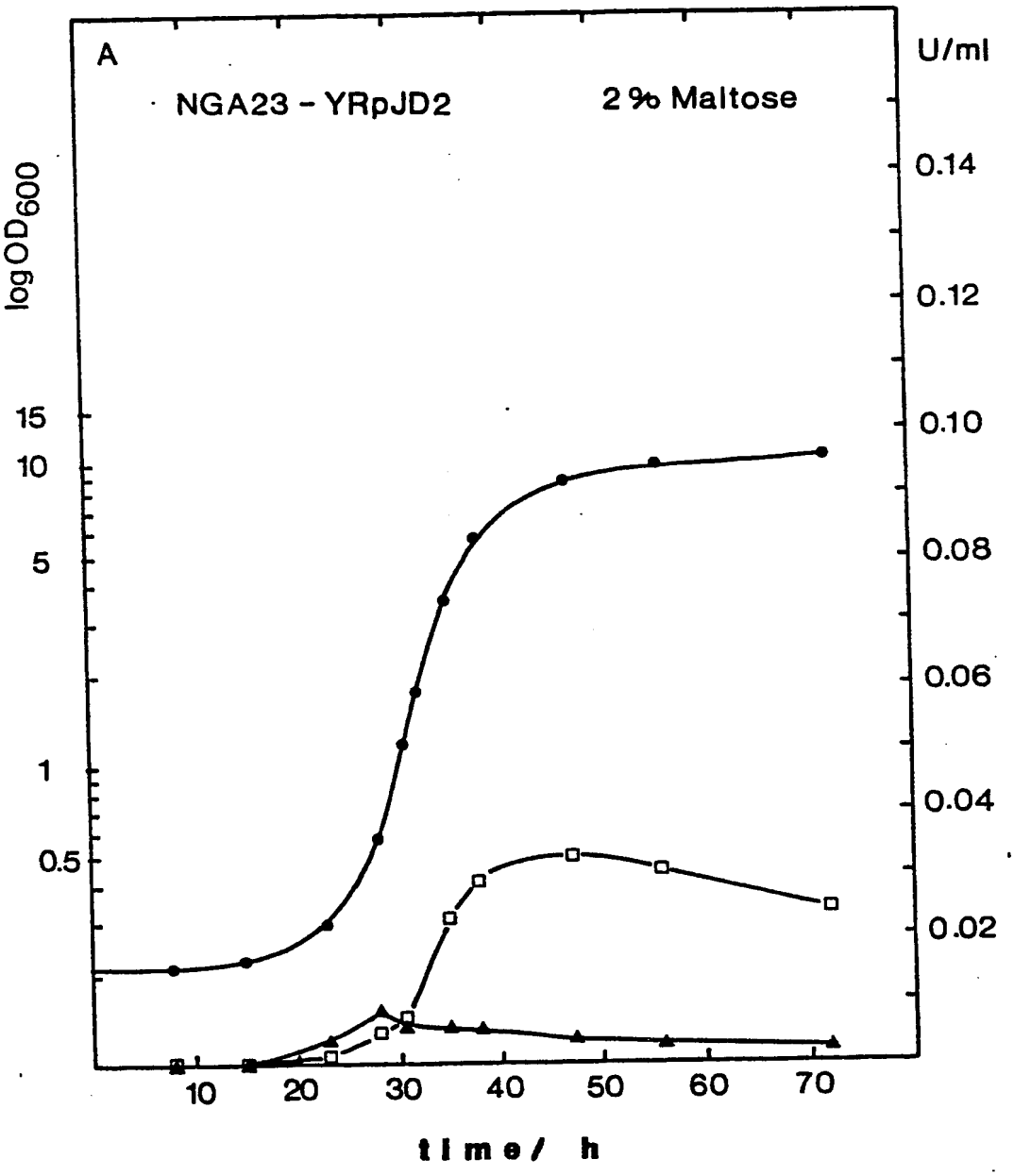

A
NGA23 - YRpJD2
2% Maltose
U/ml
log OD600
0.14
0.12
0.10
0.08
0.06
0.04
0.02
15
10
5
1
0.5
10
20
30
40
50
60
70
time/ h
cell density (log OD 600)
total α-amylase activity
specific activity (activity/OD 600)

Fig.4 (2)

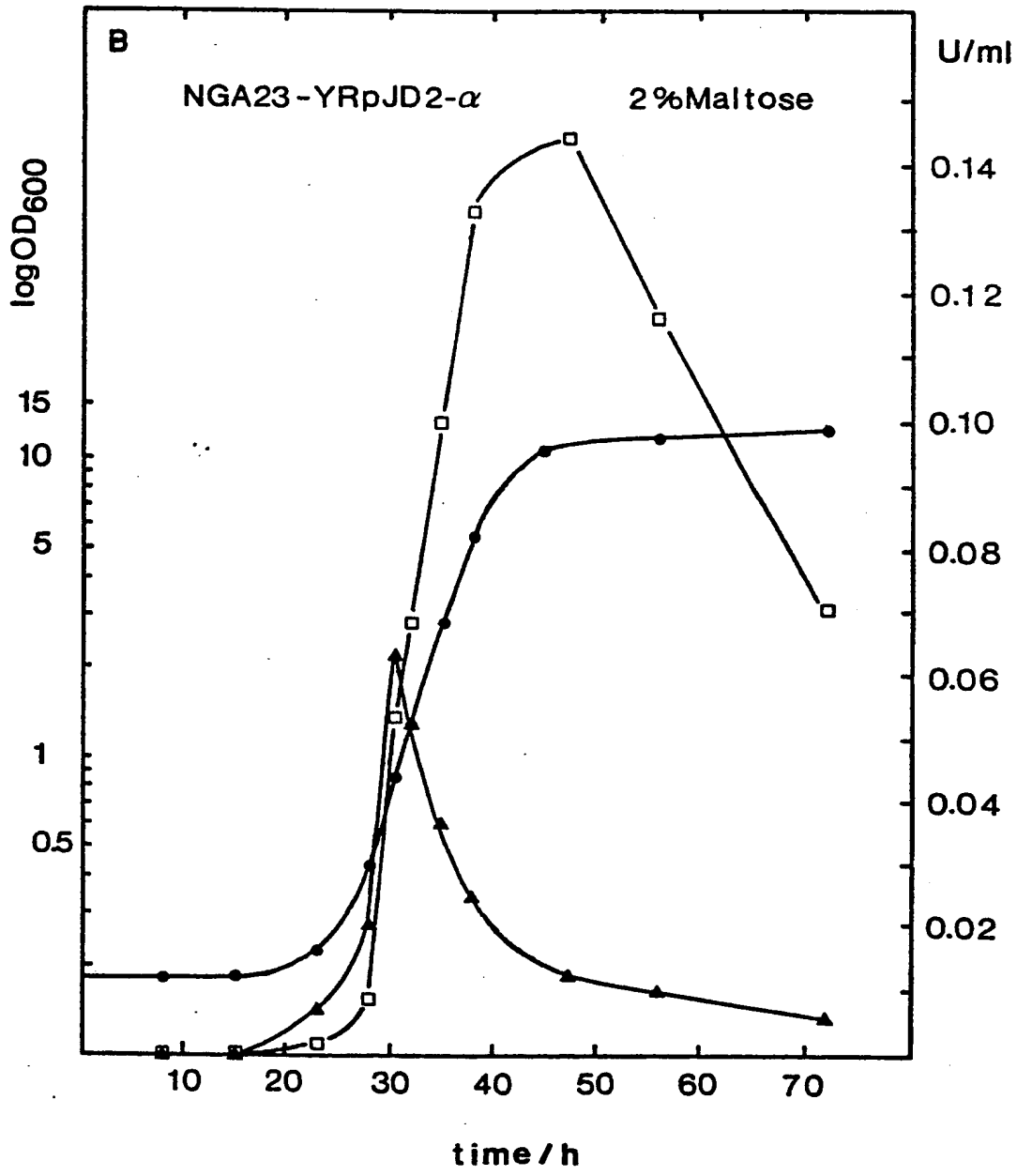
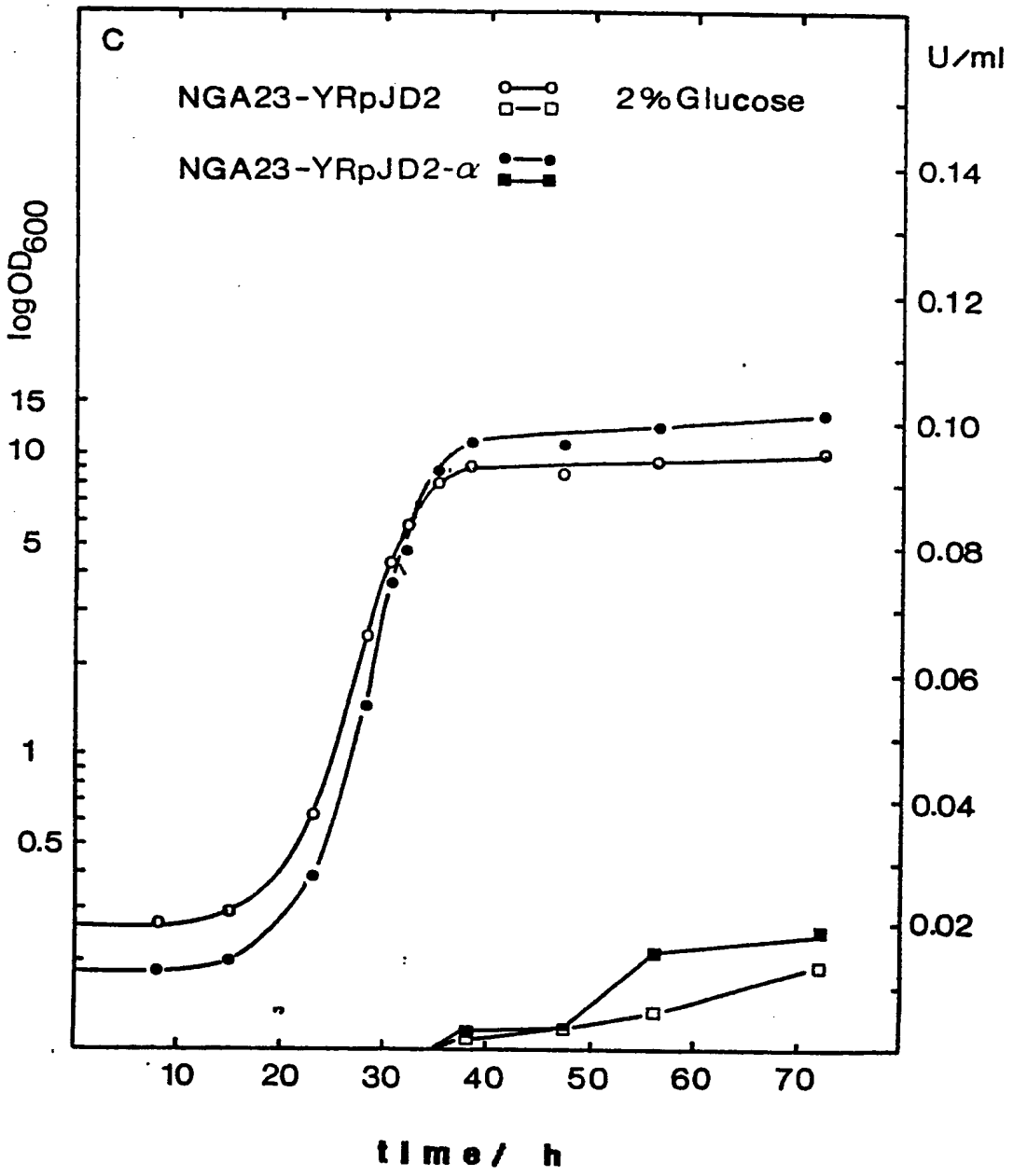

B
NGA23-YRpJD2-α
2%Maltose
U/ml
logOD600
0.14
0.12
0.10
0.08
0.06
0.04
0.02
15
10
5
1
0.5
10
20
30
40
50
60
70
time/h
cell density (log OD 600)
total α-amylase activity
specific activity (activity/OD 600)

Fig.4 (3)



cell density (log OD 600)

total α-amylase activity

Fig.4 (4)

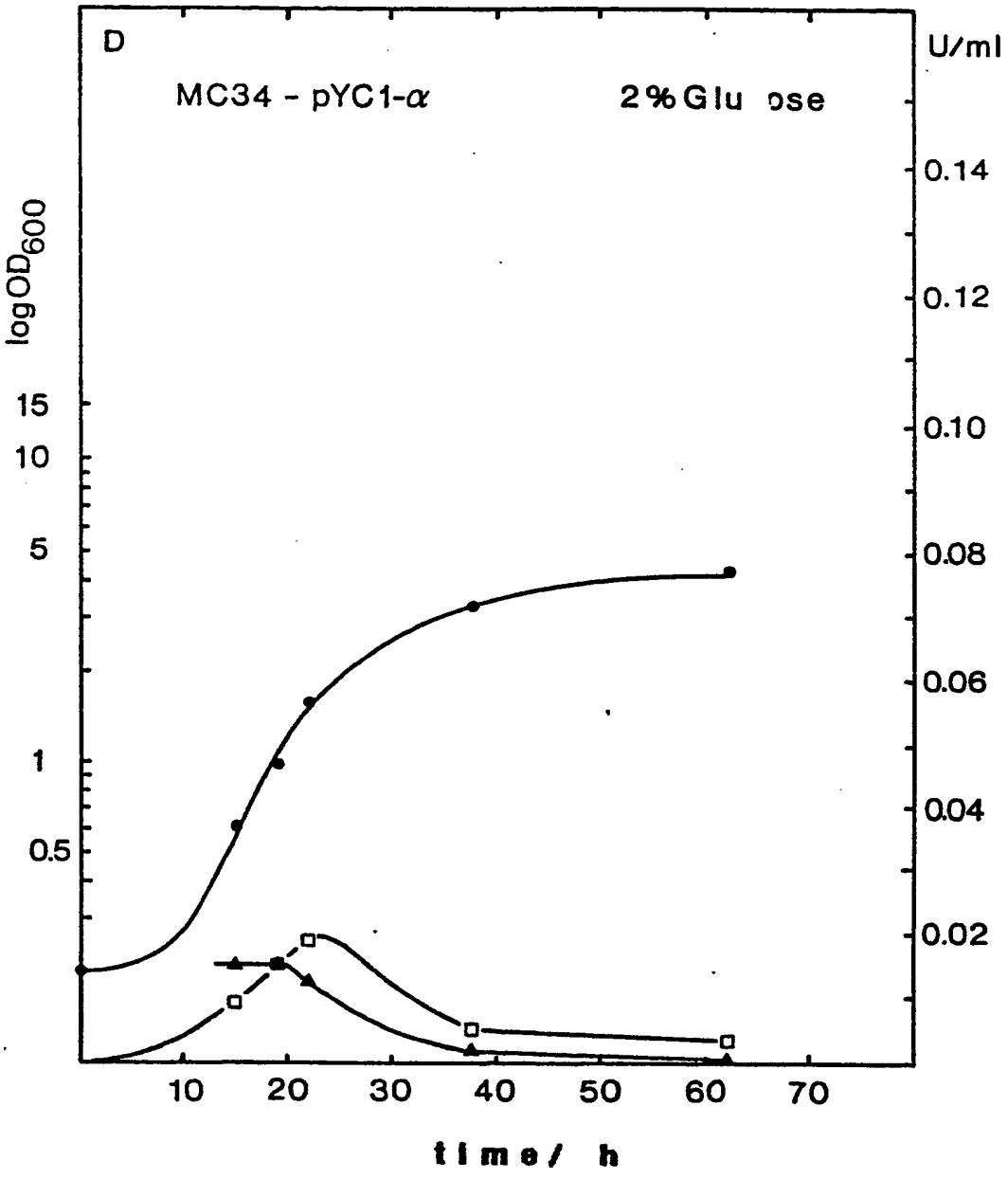
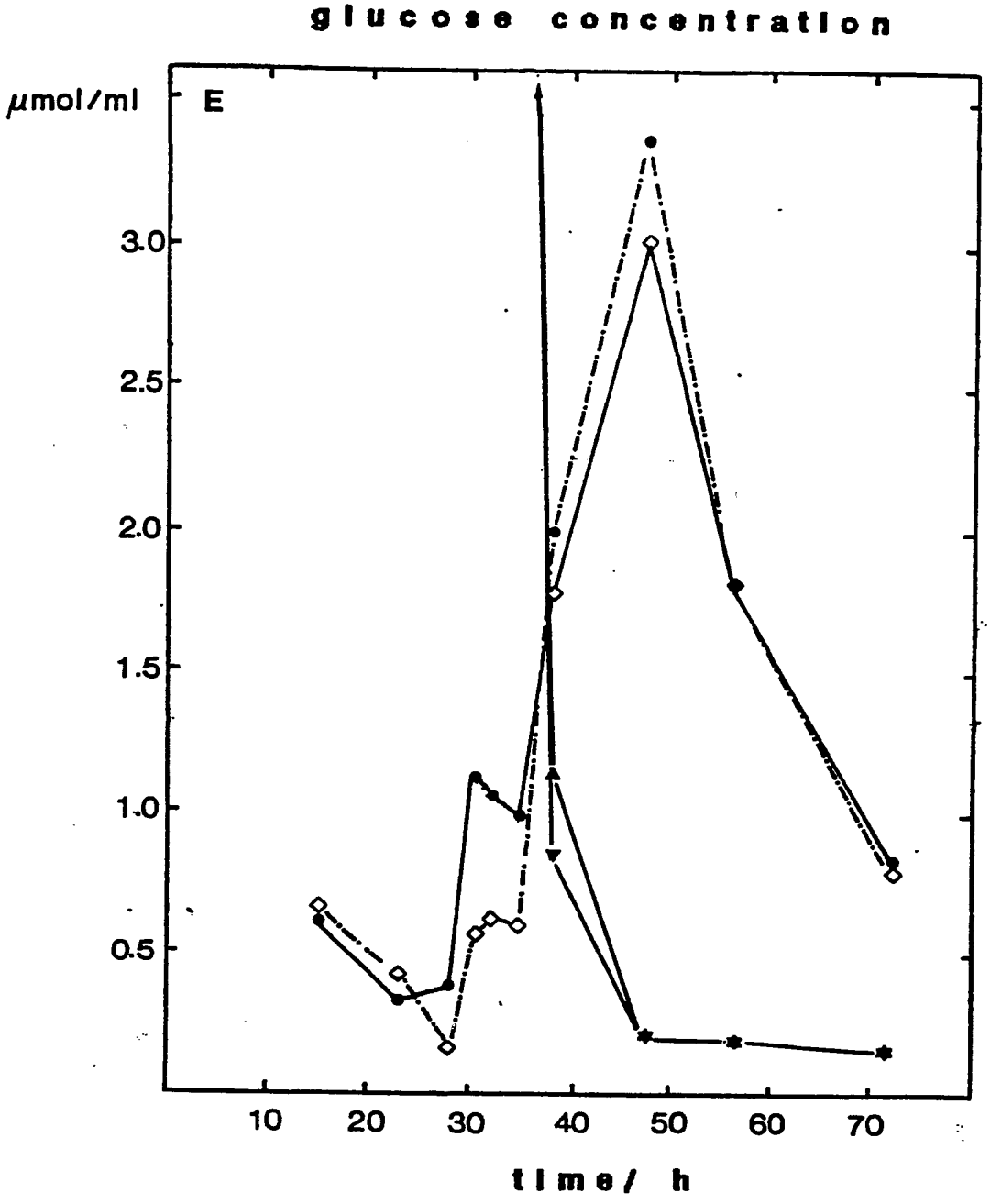

D
MC34 - pYC1-α
2% Glu ose
U/ml
0.14
0.12
0.10
0.08
0.06
0.04
0.02
logOD600
15
10
5
1
0.5
10
20
30
40
50
60
70
time/ h
cell density (log OD 600)
total α-amylase activity
specific activity (activity/OD 600)

Fig.4 (5)


glucose concentration
µmol/ml
E
3.0
2.5
2.0
1.5
1.0
0.5
10
20
30
40
50
60
70
time/ h
NGA23 - YRpJD2 , 2 % Maltose
NGA23 - YRpJD2-α , 2 % Maltose
NGA23 - YRpJD2 , 2 % Glukose
NGA23 - YRpJD2-α , 2 % Glukose

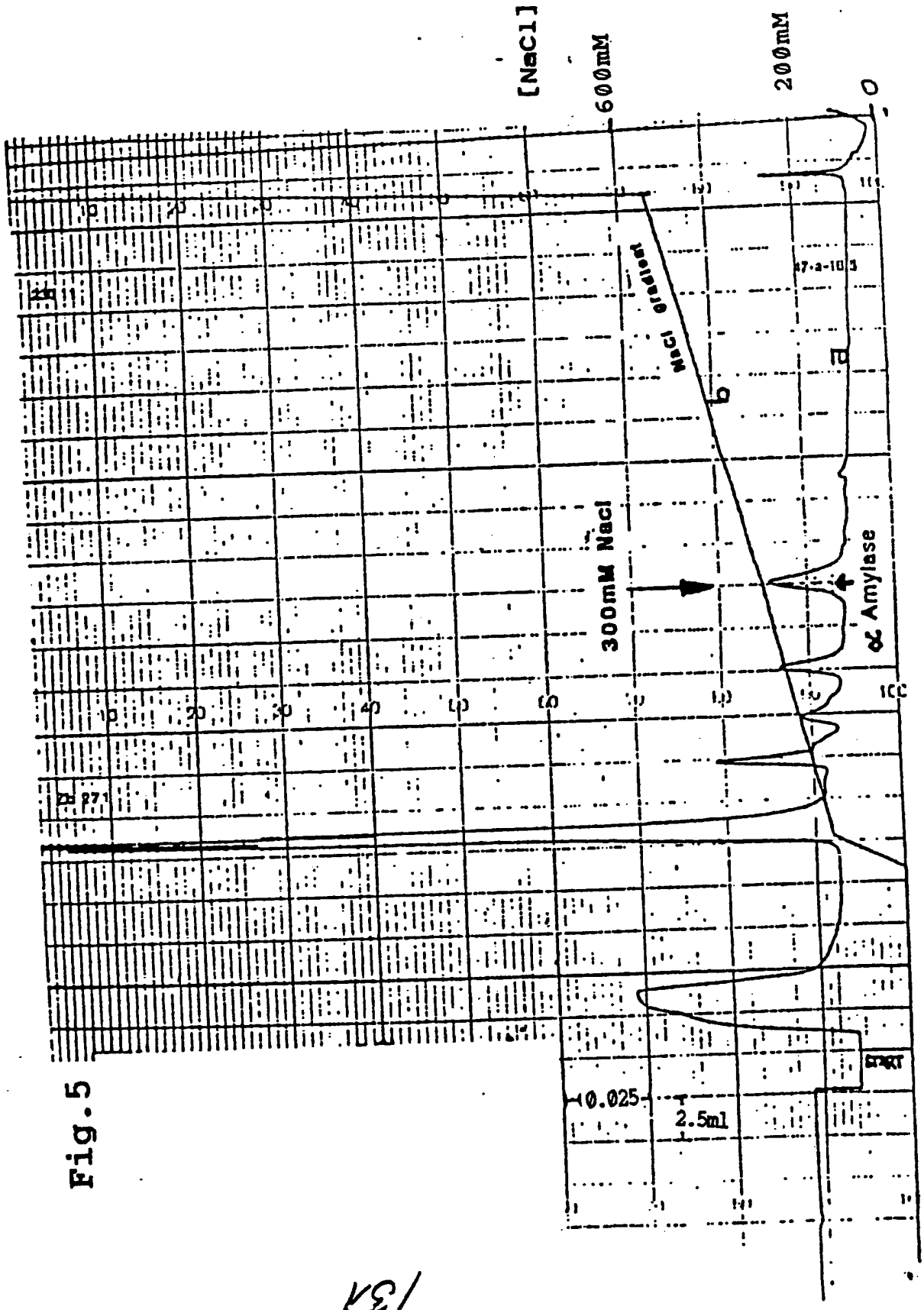

[NaCl]
600mM
200mM
0
NaCl gradient
300mM NaCl
α Amylase
0.025
2.5ml

Fig.5

Fig.6

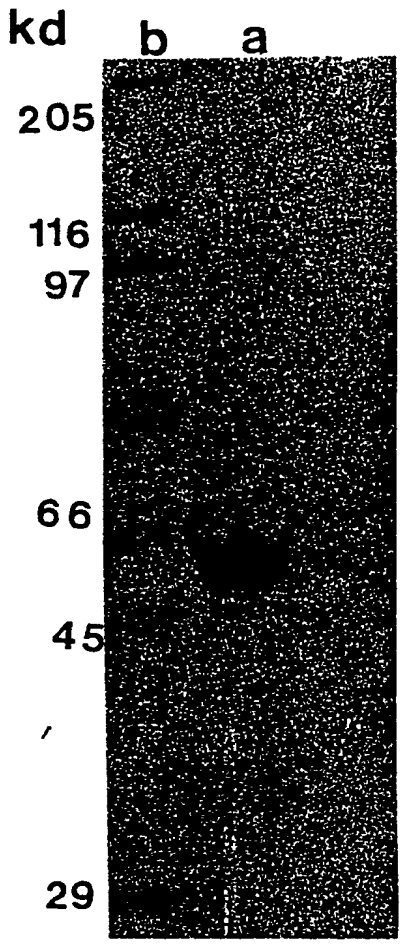


Fig 7

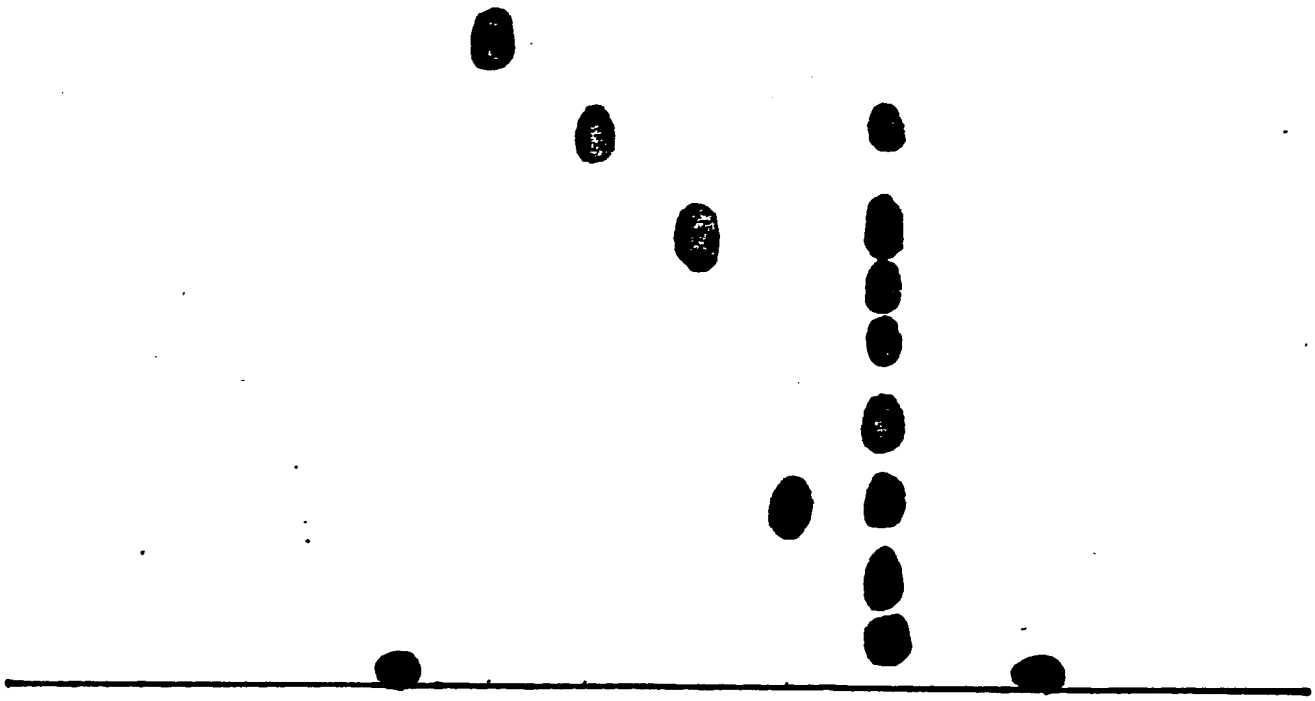

extracellular medium of GRF 18 + soluble starch
extracellular medium of GRF 18:pYc10c + soluble starch
maltoheptulose
maltotriose
maltose
glucose
soluble starch

Fig.8(1)

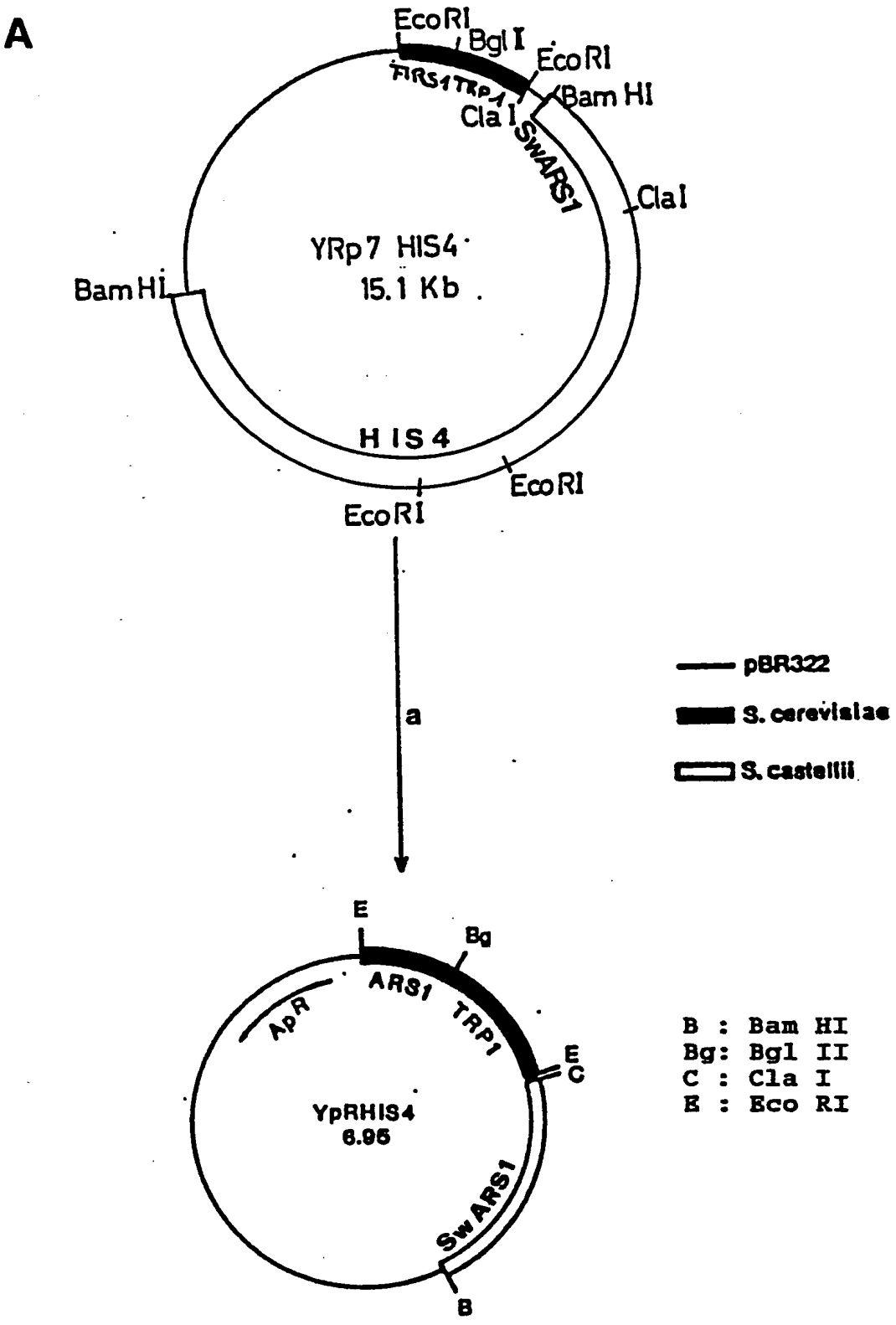

A
EcoRI
Bgl I
EcoRI
FIRS1TRP1
Cla I
Bam HI
SwARS1
ClaI
YRp7 HIS4
15.1 Kb
BamHI
HIS4
EcoRI
EcoRI
a
pBR322
S. cerevisiae
S. castellii
E
Bg
ARS1
TRP1
ApR
E
C
YpRHIS4
6.95
SwARS1
B
B : Bam HI
Bg: Bgl II
C : Cla I
E : Eco RI

Fig.8 (2)

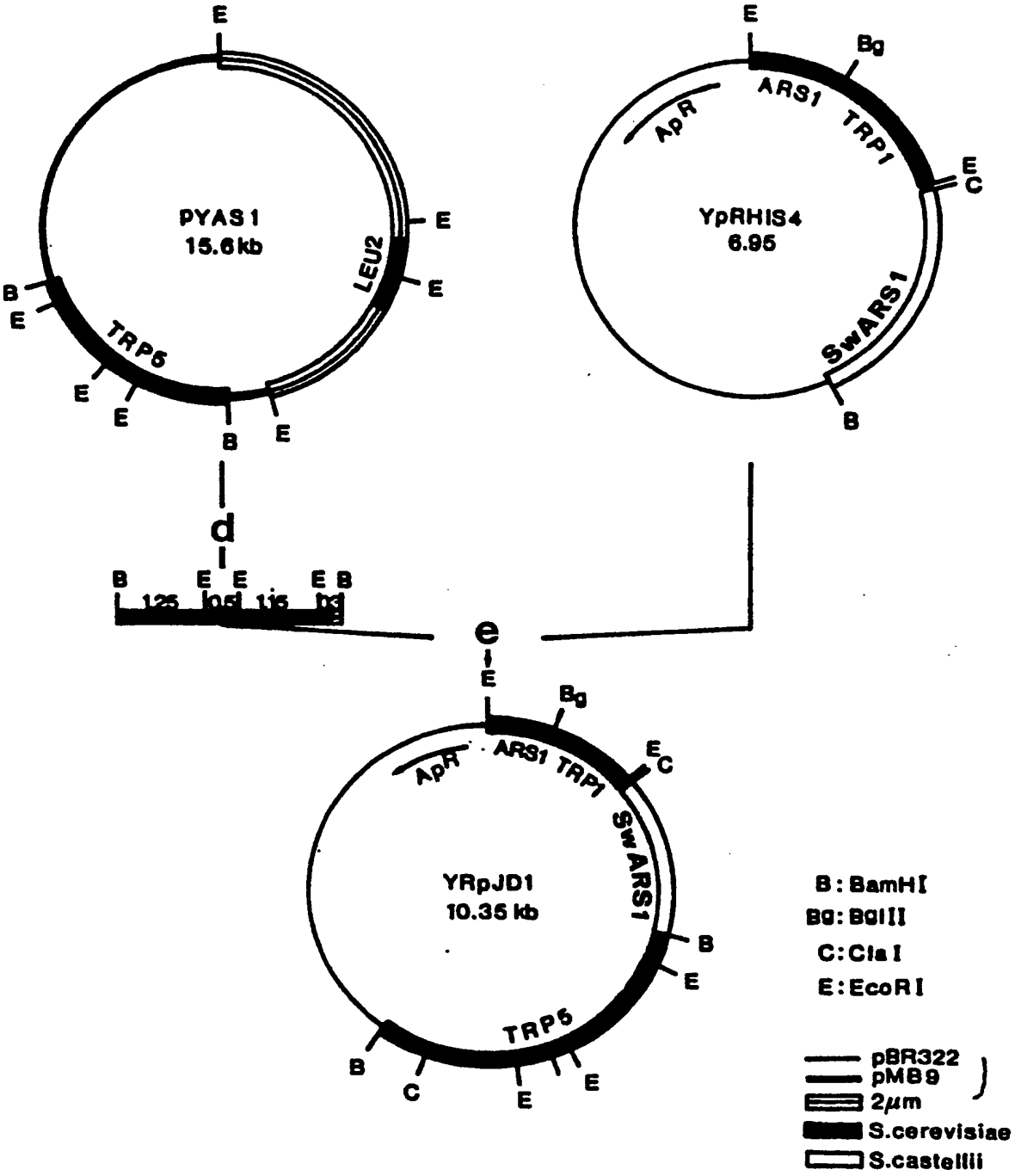
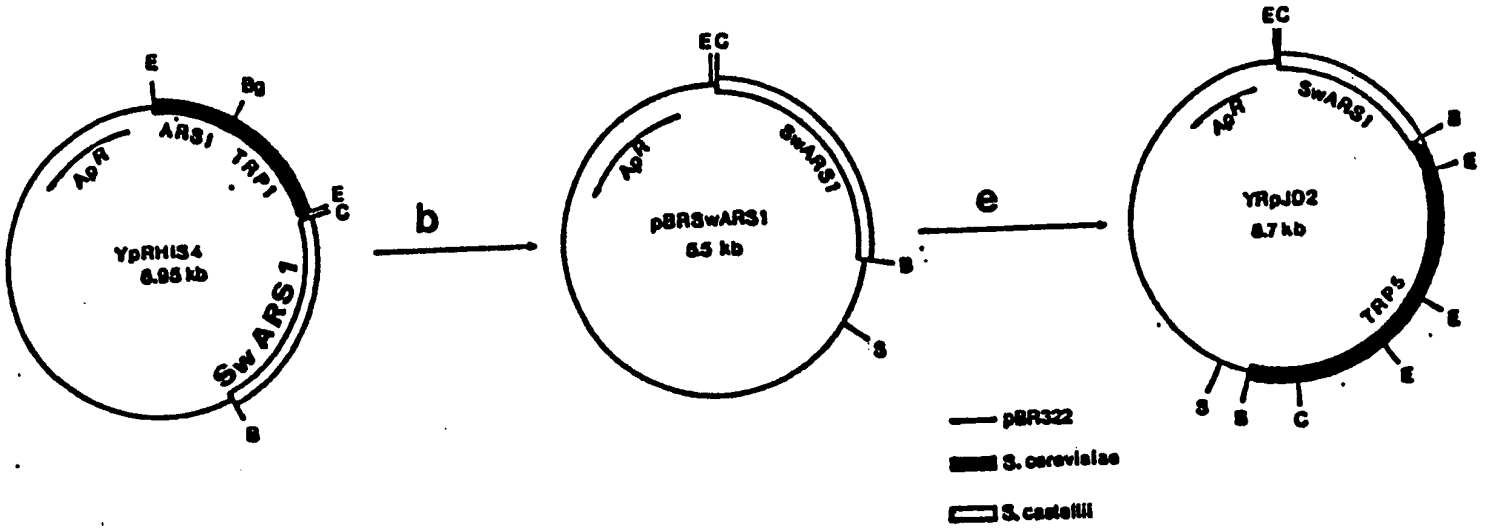

PYAS1
15.6kb
LEU2
TRP5
YpRHIS4
6.95
ApR
ARS1
TRP1
SwARS1
YRpJD1
10.35 kb
B : BamHI
Bg: BglII
C: ClaI
E: EcoRI
pBR322
pMB9
2μm
S.cerevisiae
S.castellii

Fig.8 (3)/A



B: Bam HI Bg: Bgl II C: Cla I S: Sal I

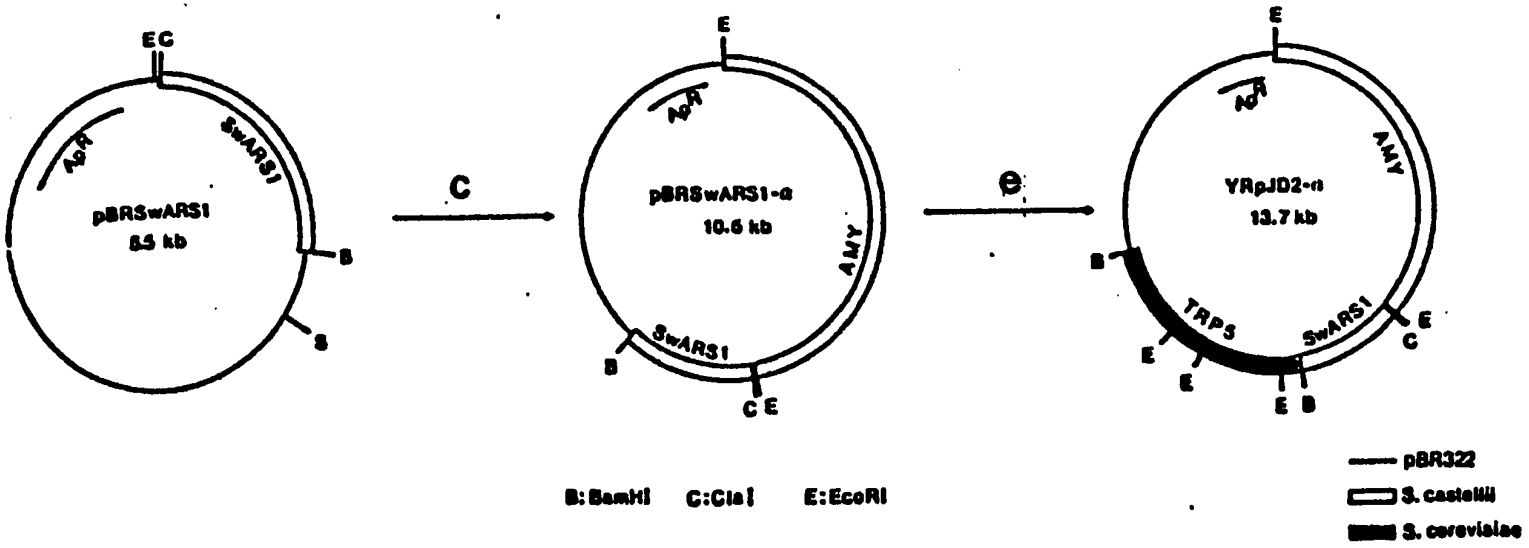
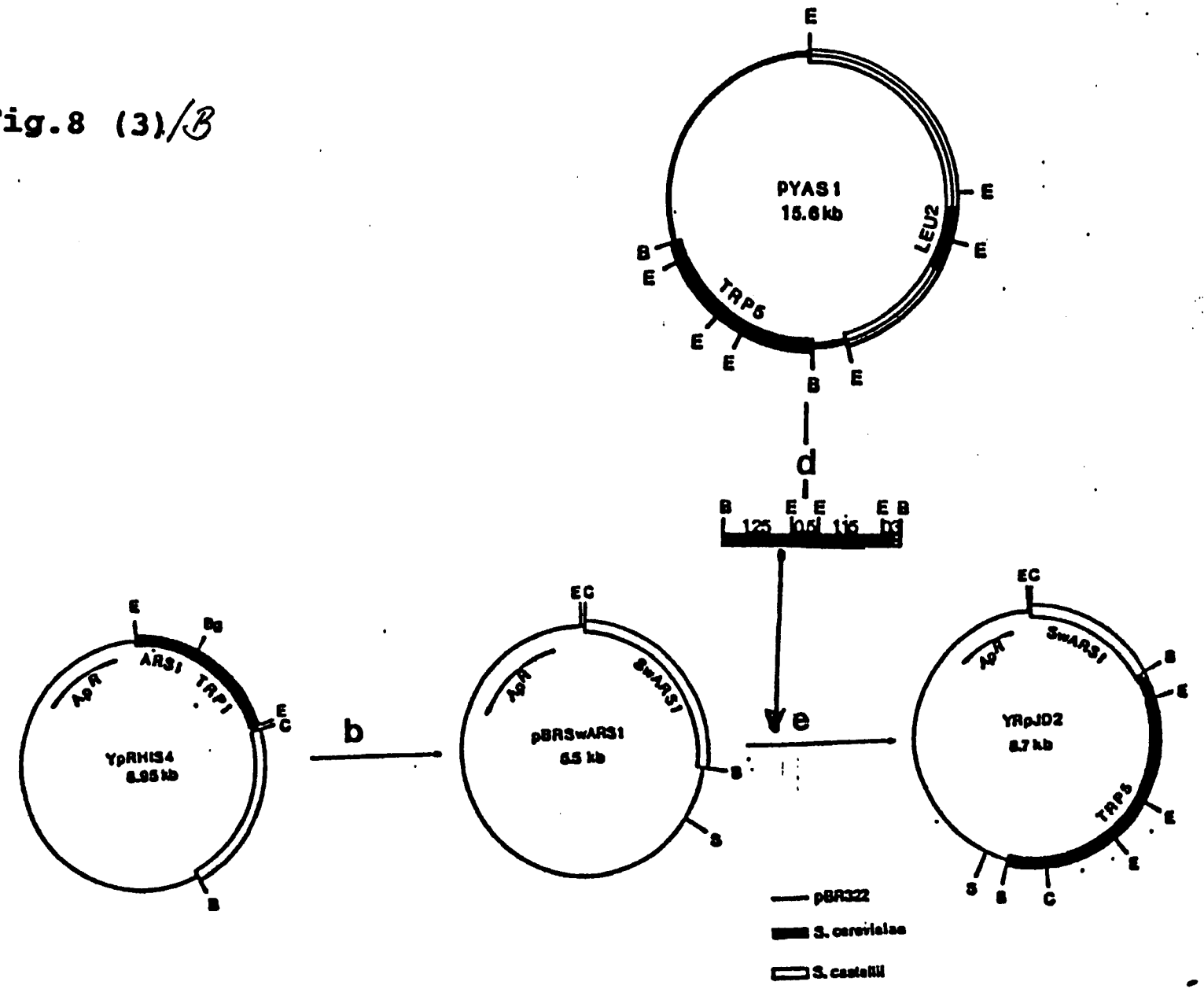
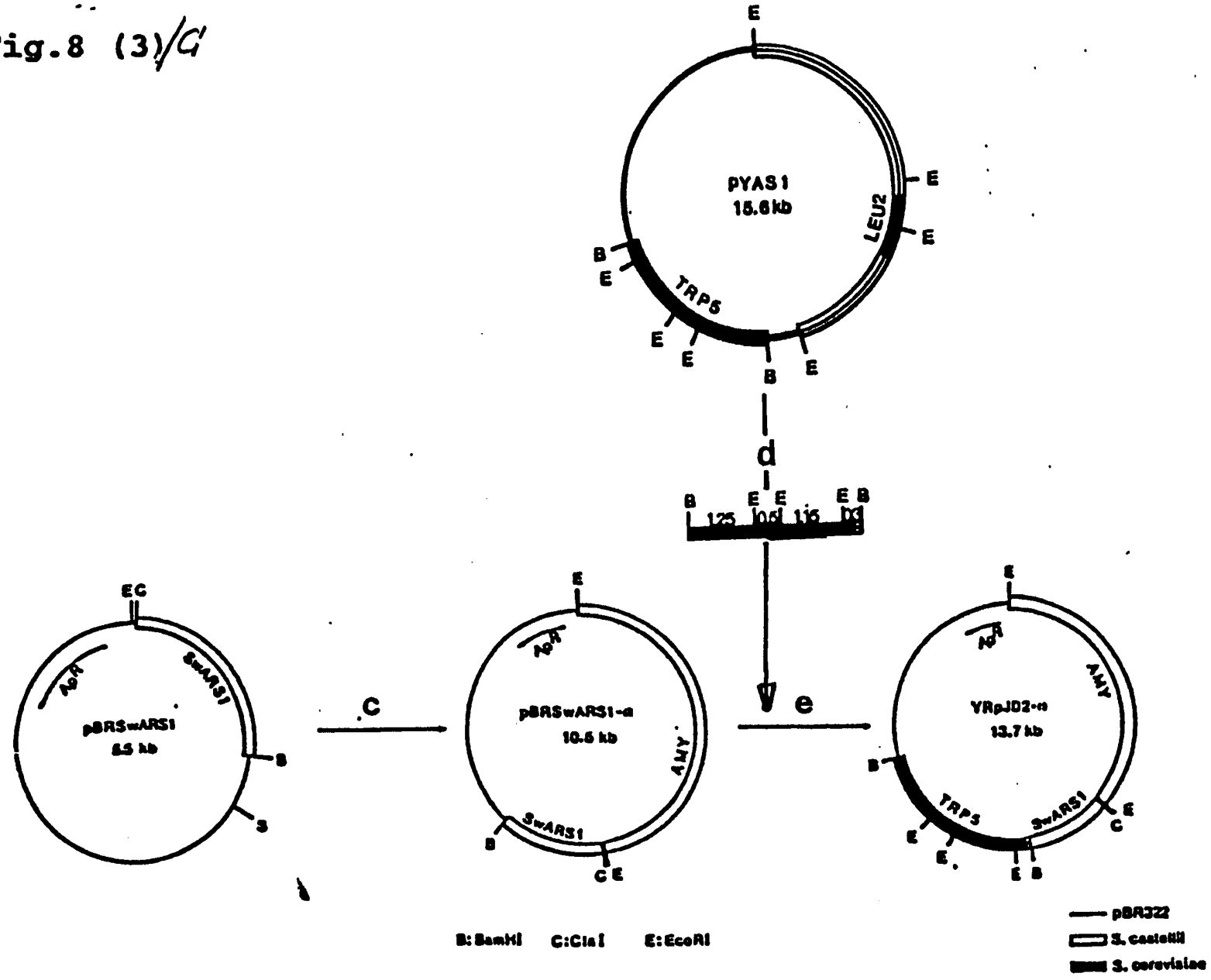


Fig.8 (3)/B

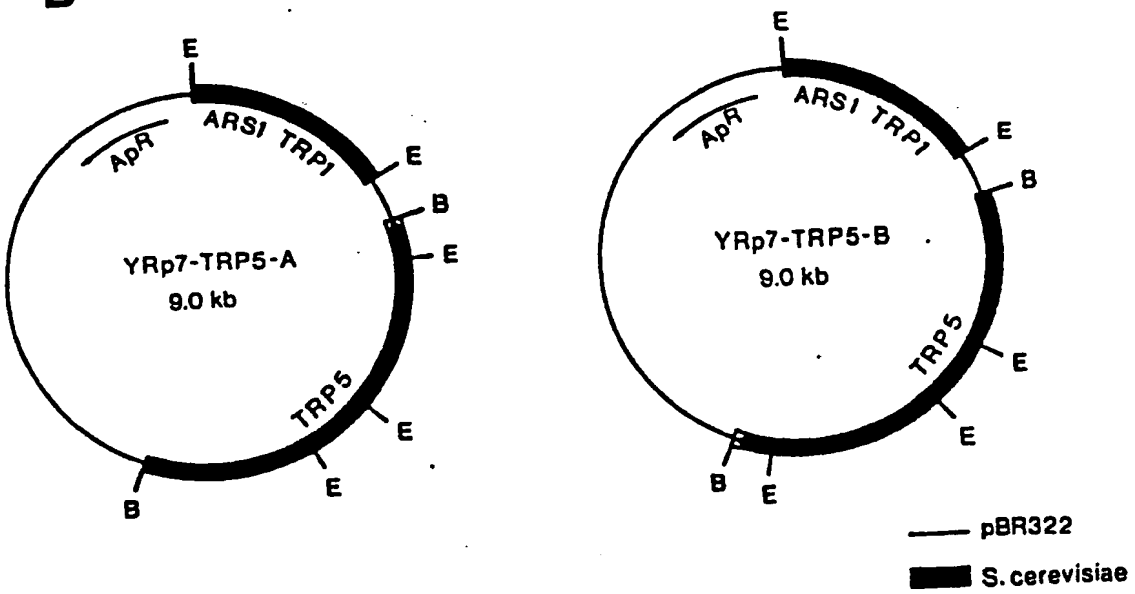


B: Bam HI Bg: Bgl II C: Cla I S: Sal I

Fig.8 (3)/C

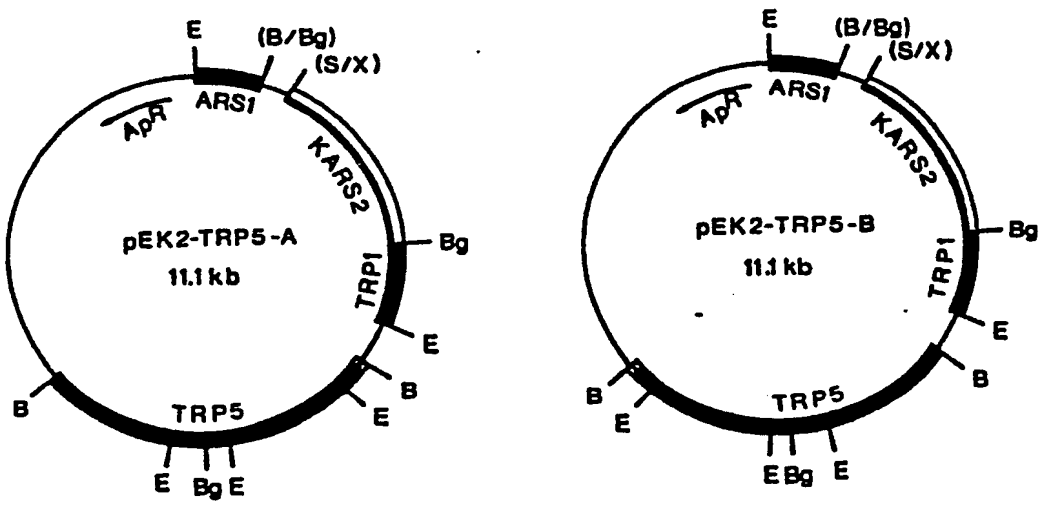

pYAS1
15.6kb
LEU2
TRP5
pBRSwARS1
8.5 kb
SwARS1
Ap^R
pBRSwARS1-a
10.6 kb
AMY
YRpJD2-a
13.7 kb
TRP3
c
d
e
B: BamHI C: ClaI E: EcoRI
pBR322
S. castellii
S. cerevisiae

B



E: EcoR I B: BamH I

C



B: Bam HI Bg: Bgl II C: Cla I S: Sal I
X: Xba I

Fig.9 (1)

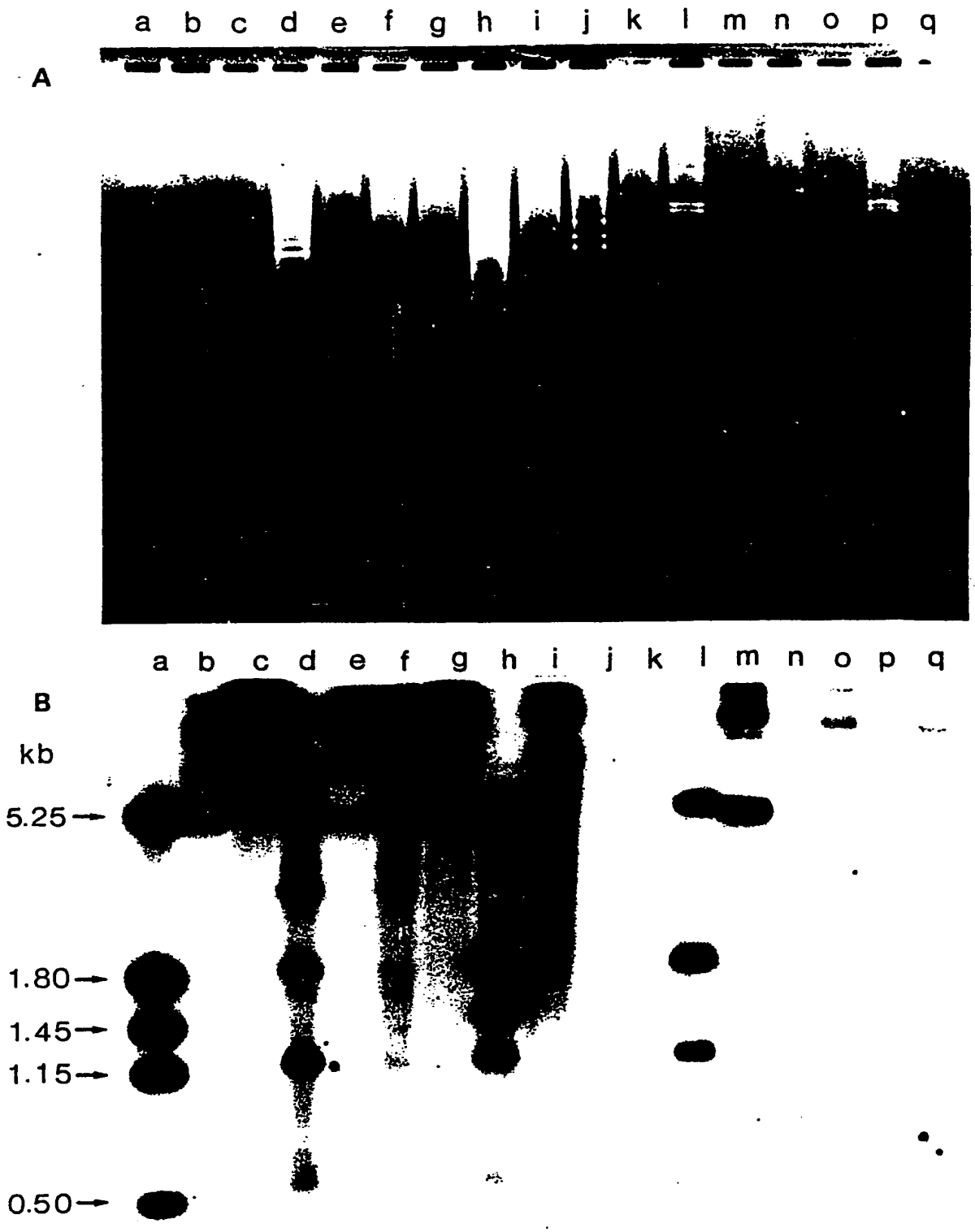

a b c d e f g h i j k l m n o p q
A
a b c d e f g h i j k l m n o p q
B
kb
5.25
1.80
1.45
1.15
0.50

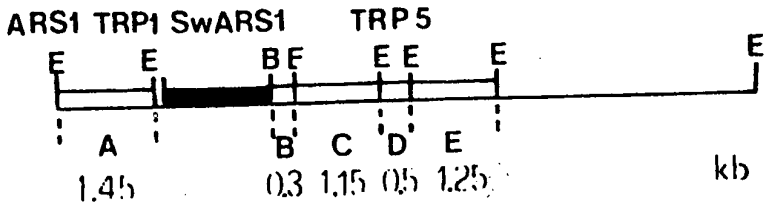
Fig.9 (2)

C



D

Hybridizingprobe : YRpJD1



0 257 115

Fig.10 (1)

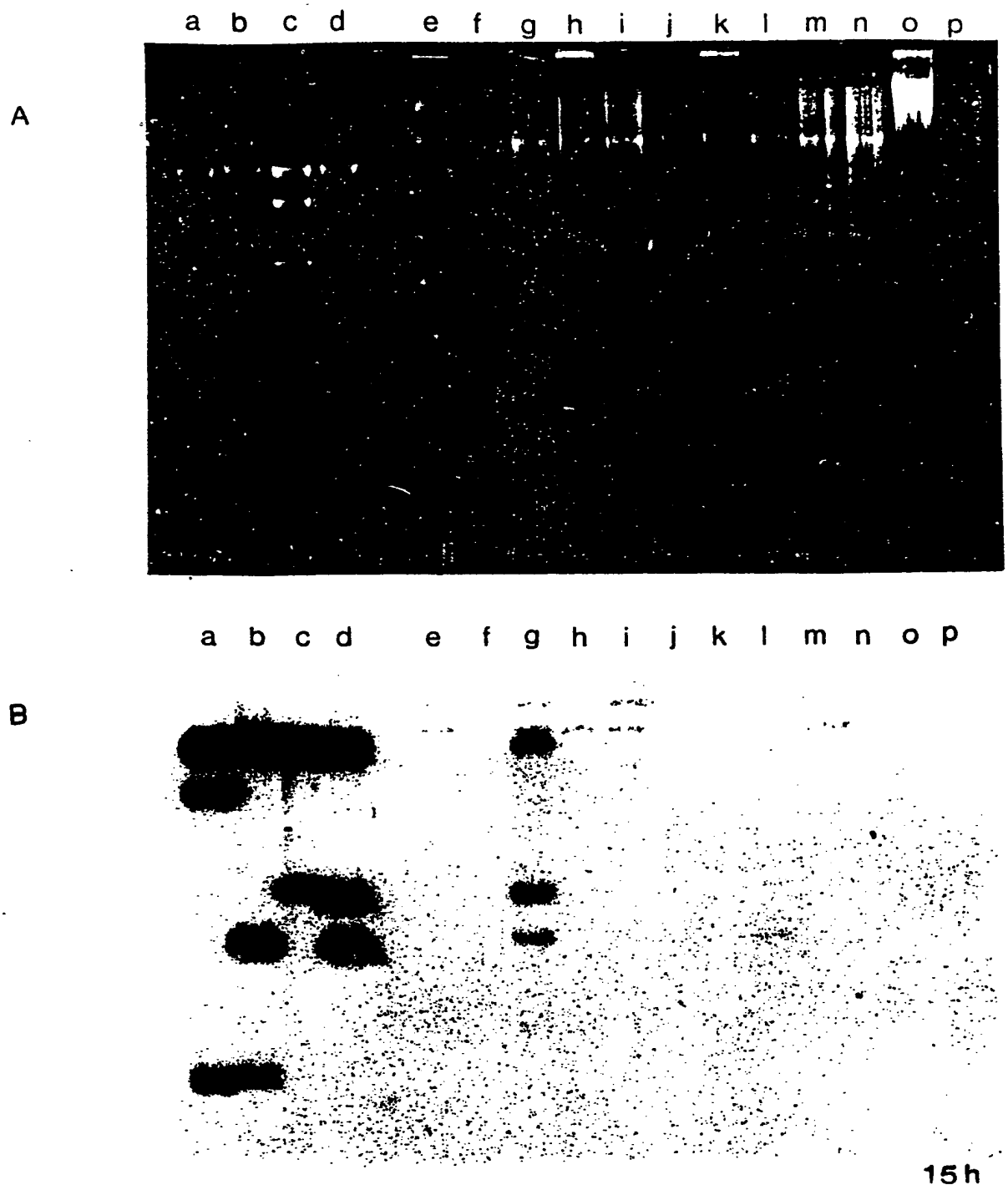
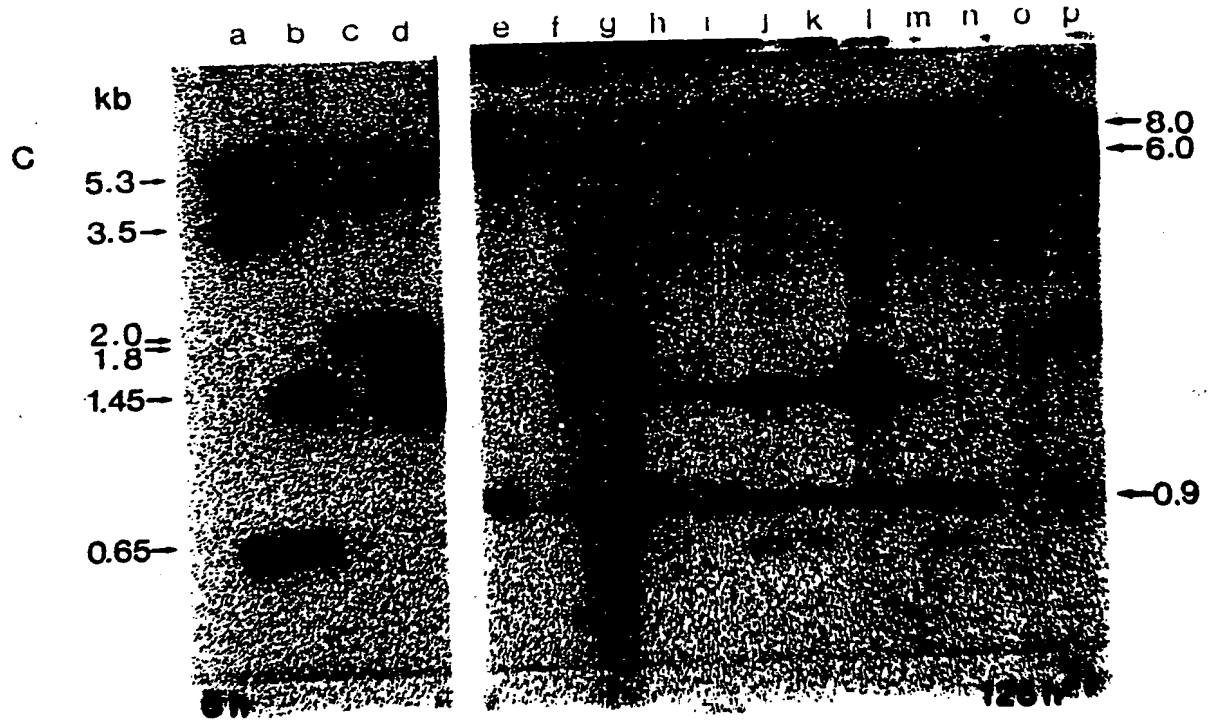


Fig.10 (2)


C
a b c d
e f g h i j k l m n o p
kb
5.3
3.5
2.0
1.8
1.45
0.65
8.0
6.0
0.9

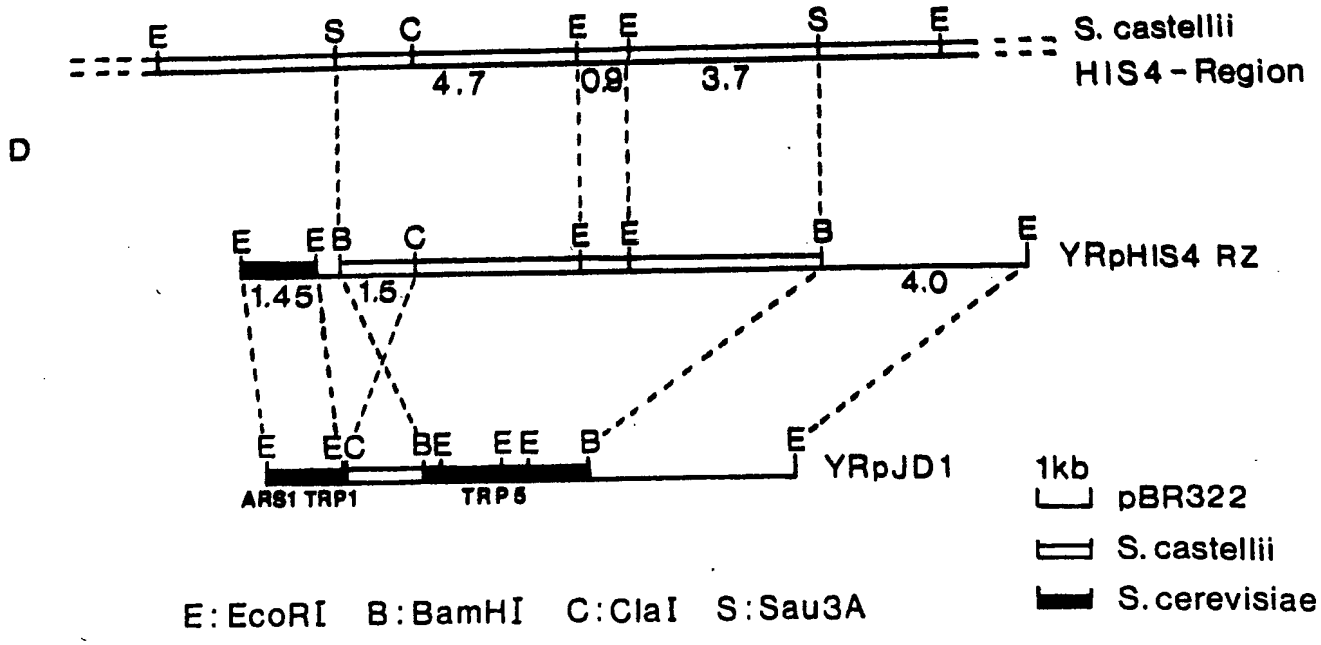
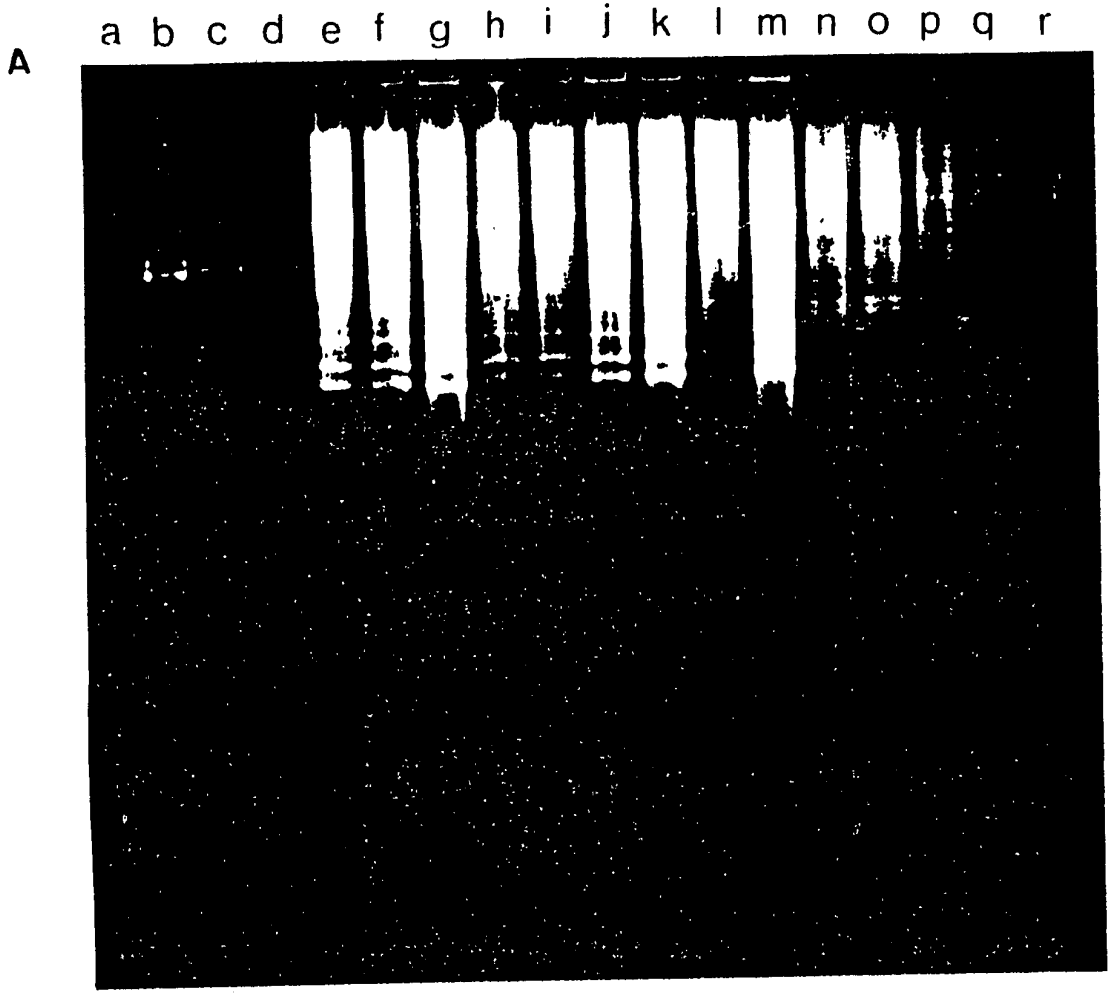

D
E S C E E S E
S. castellii
HIS4 - Region
4.7
0.9
3.7
E E B C E E B E
YRpHIS4 RZ
1.45
1.5
4.0
E EC BE EE B E
YRpJD1
ARS1 TRP1
TRP5
1kb
pBR322
S. castellii
S. cerevisiae
E: EcoRI B: BamHI C: ClaI S: Sau3A

Fig.11 (1)



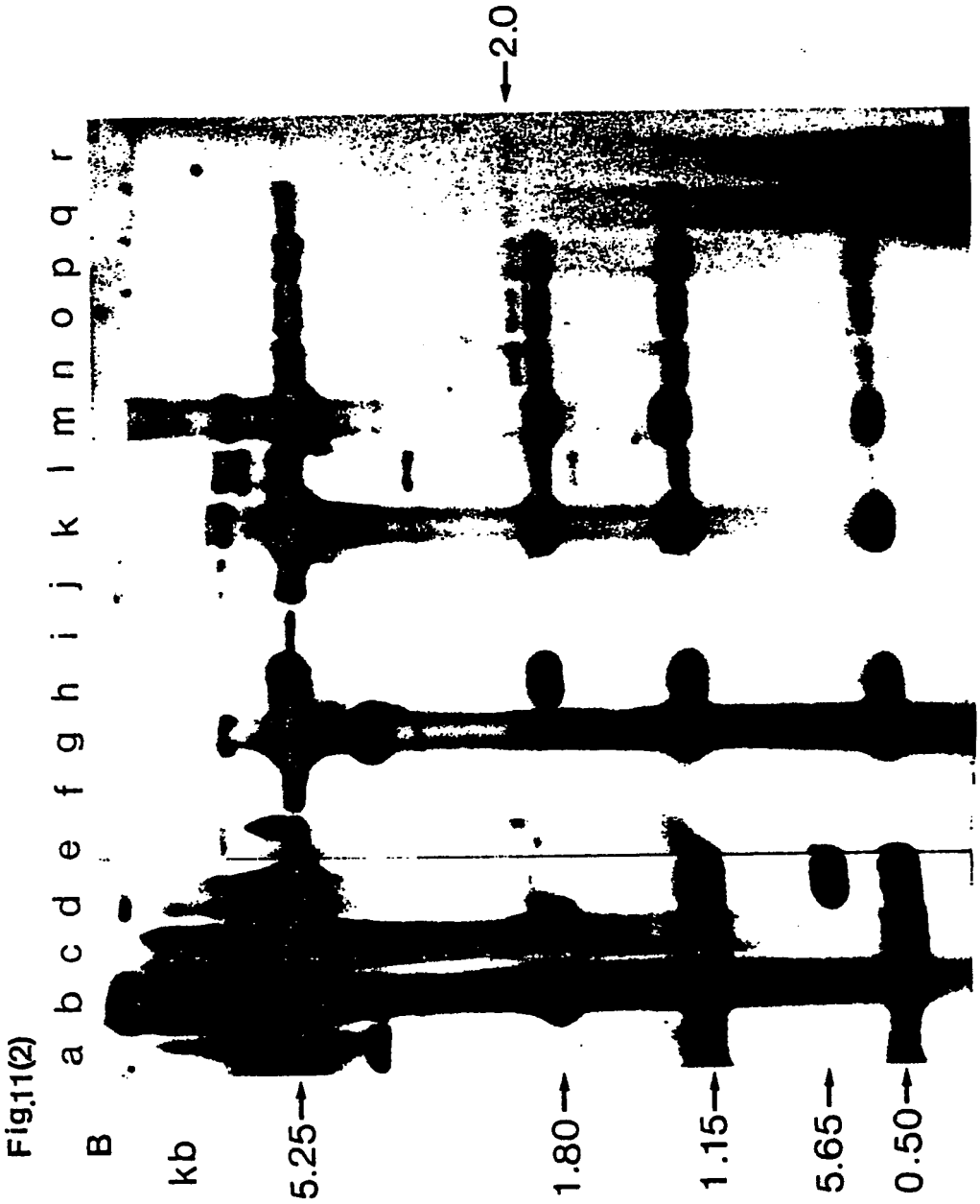


Fig.11(2)

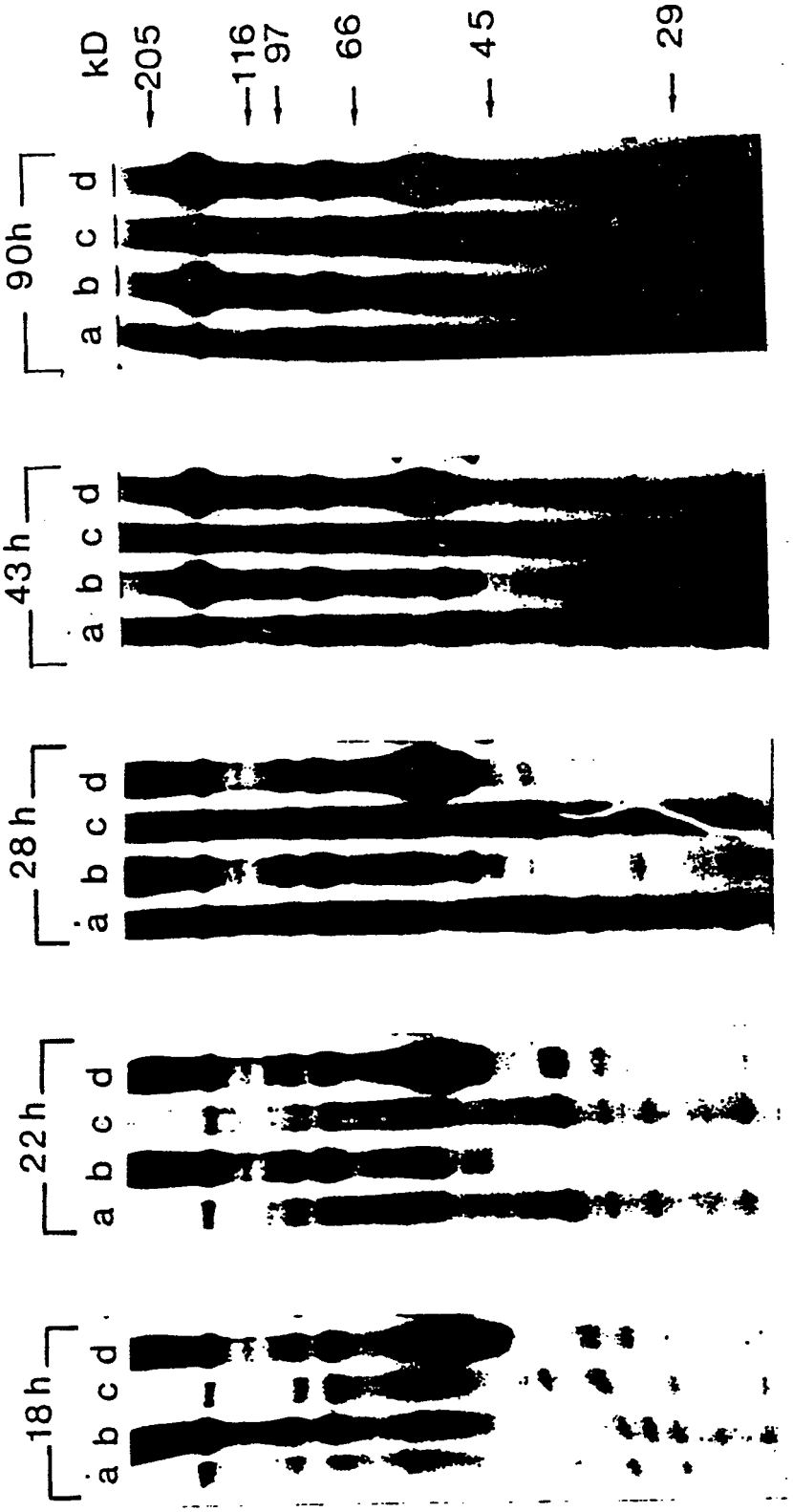
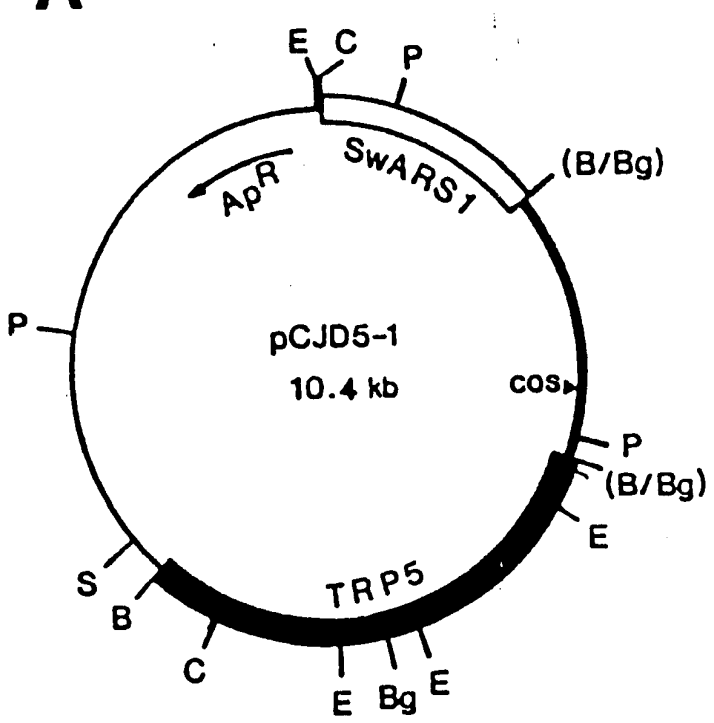
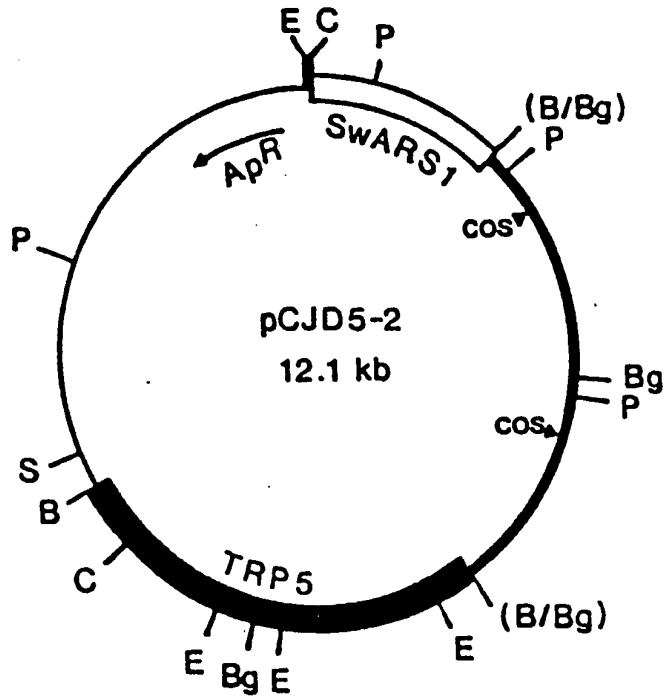


Fig.12

Fig.13 (1)

A





B: BamHI
Bg: Bgl II
C: Cla I
E: EcoR I
P: Pvu II
S: Sal I

pBR322
λ
S. castellii
S. cerevisiae

0 257 115

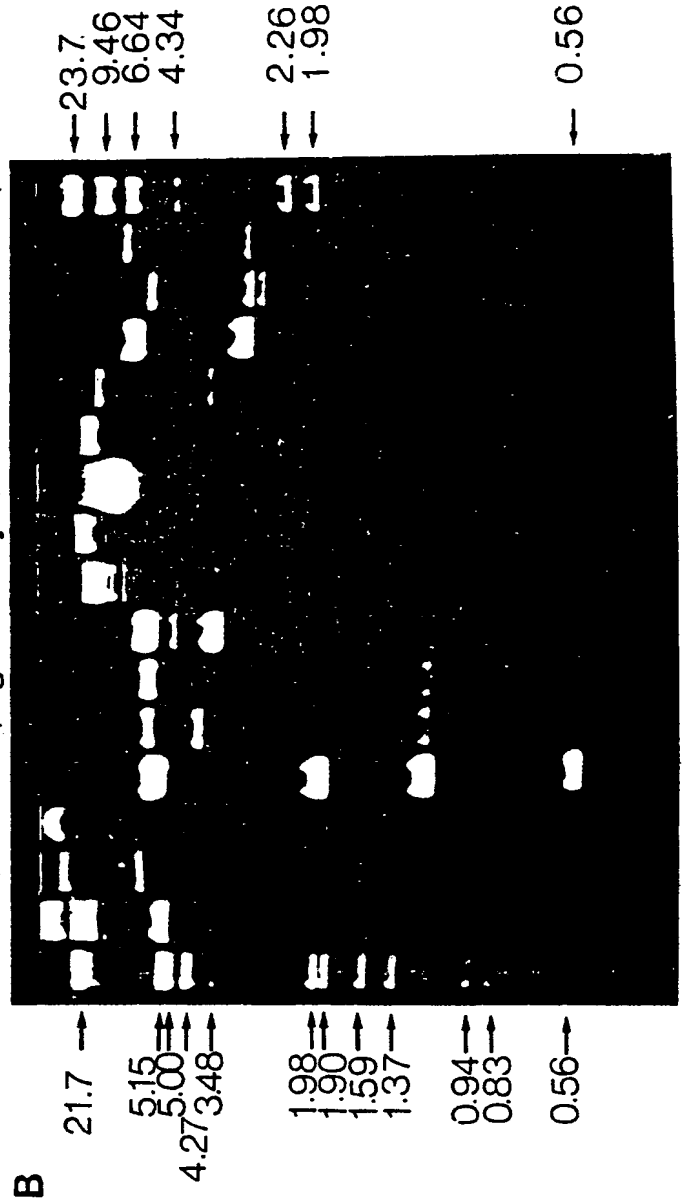

B
a b c d e f g h i j k l m n o p q
23.7
9.46
6.64
4.34
2.26
1.98
0.56
21.7
5.15
5.00
4.27
3.48
1.98
1.90
1.59
1.37
0.94
0.83
0.56

Fig.13 (2)

Fig.14

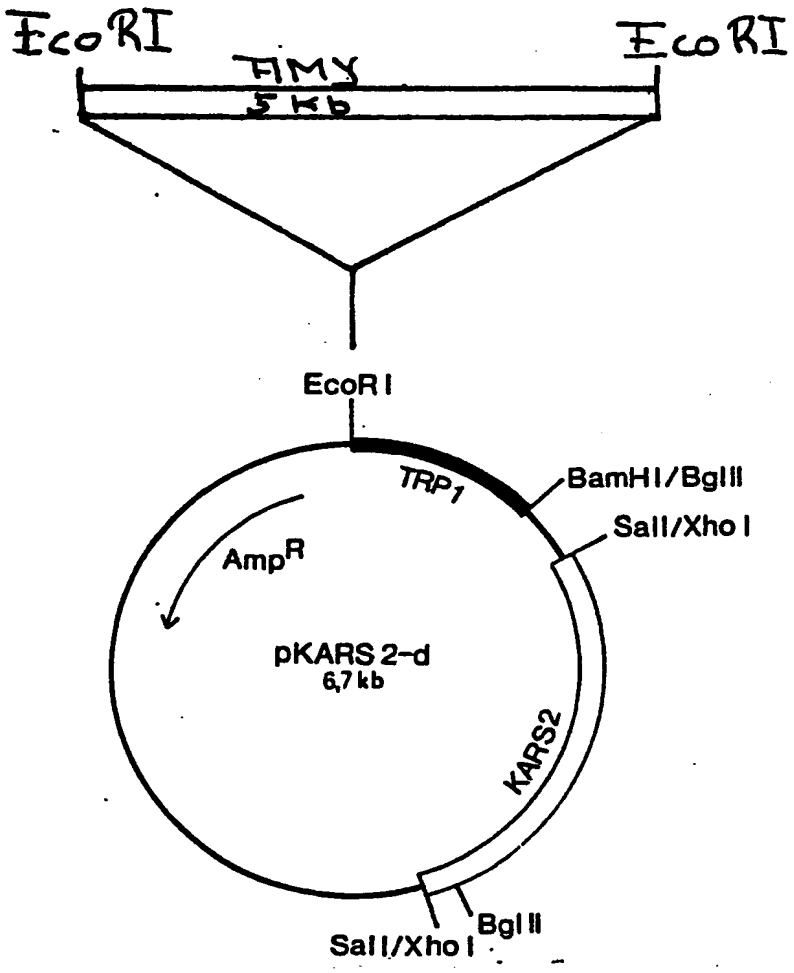
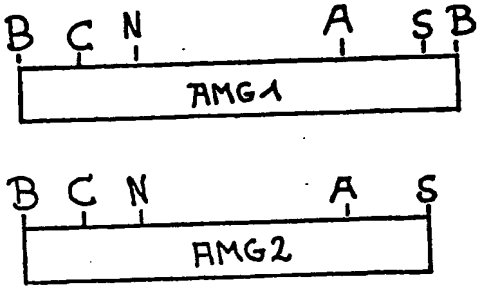

EcoRI
AMY
5 kb
EcoRI
EcoR I
TRP1
BamHI/BglII
SalI/Xho I
AmpR
pKARS 2-d
6,7 kb
KARS2
SalI/Xho I
BglII

Fig.15 (1)



A : Ava I
Bg :Bgl II
B :Bam HI
E ;Eco RI
N :Nru I
P ;Pvu II
Ps :Pst I
S :Sal I
X :Xho I

pBR 322
Saccharomyces cerevisiae
Kluyveromyces lactis
Schwanniomyces castellii
2 μ DNA
λ DNA

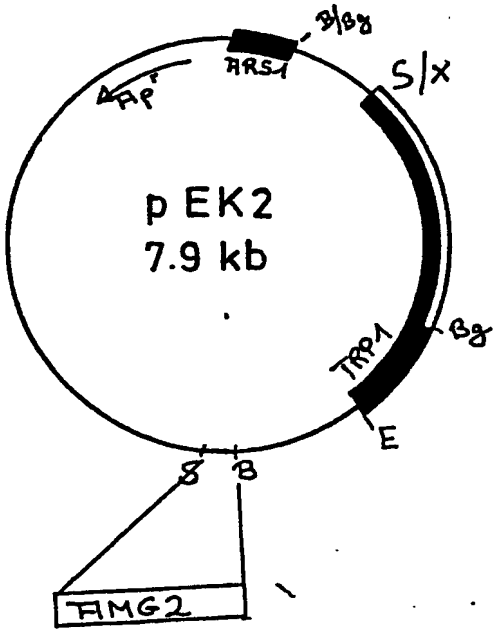
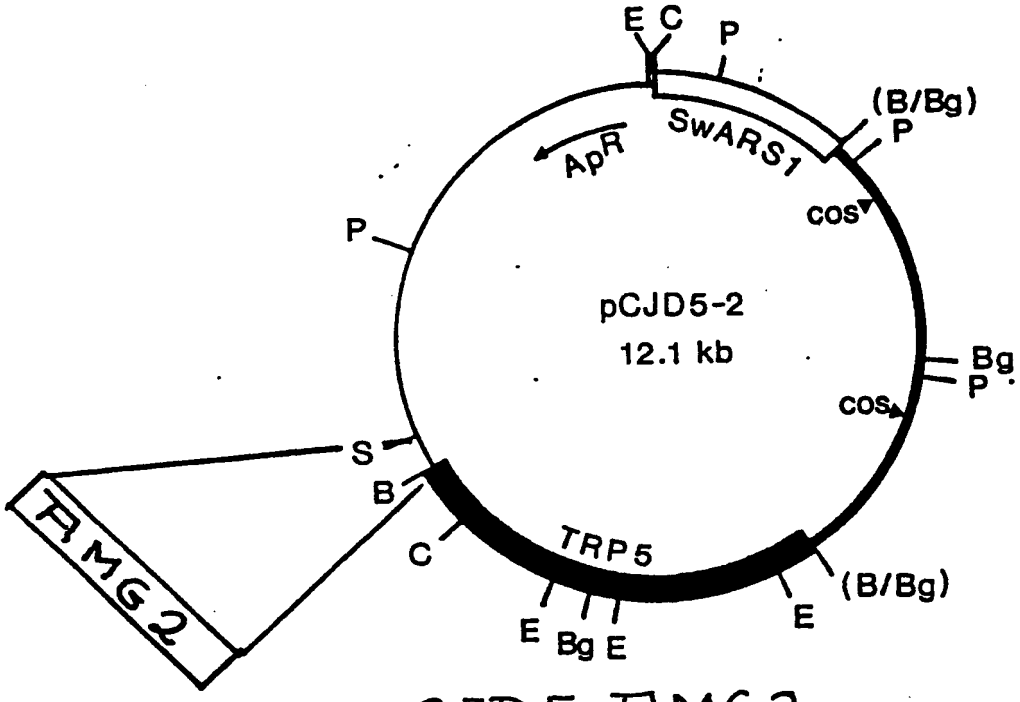
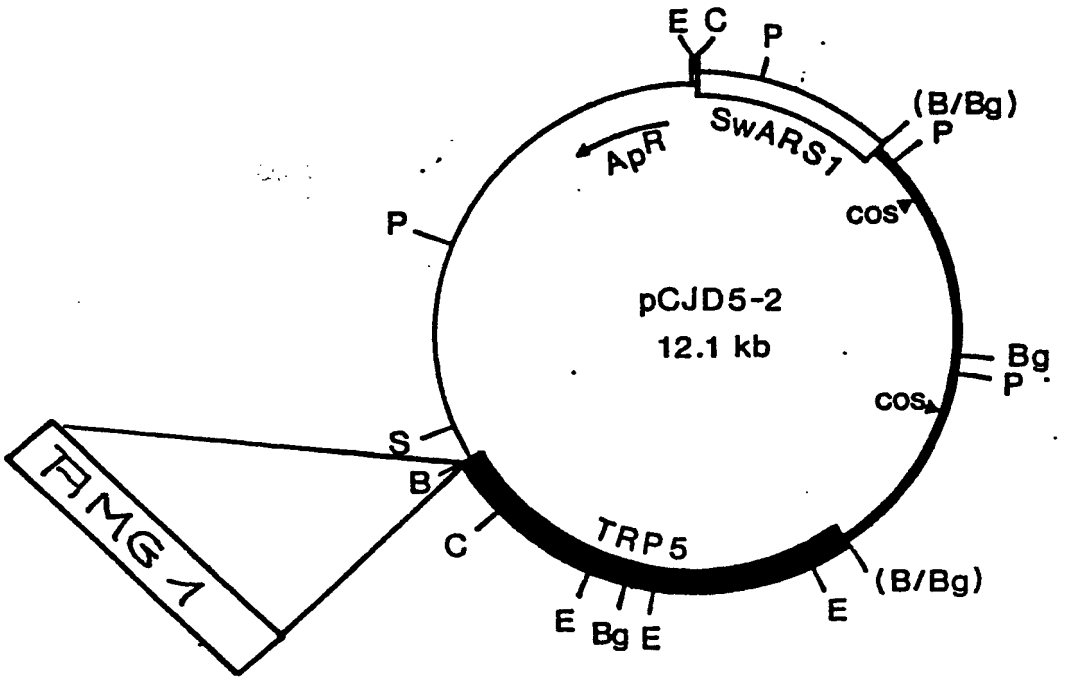


Fig.15 (2)

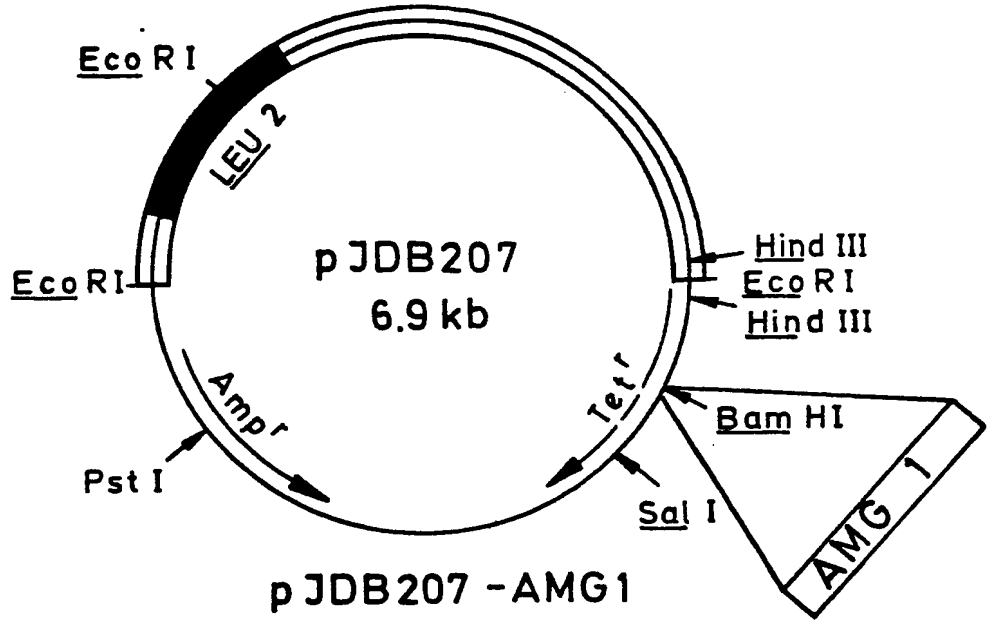


pCJD5-AMG2

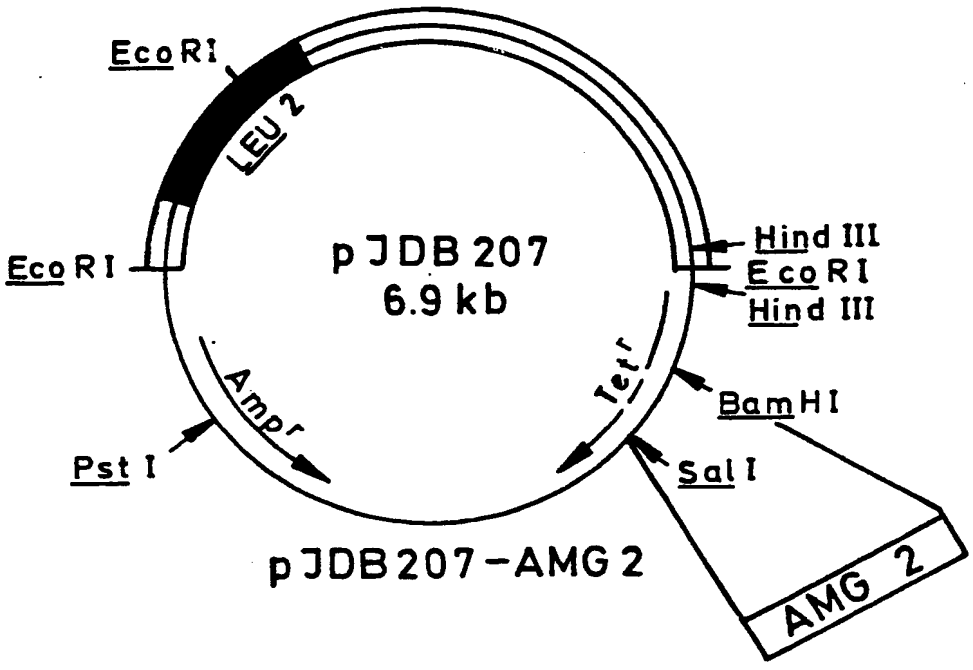


pCJD5-AMG1

FIG. 15 (3)

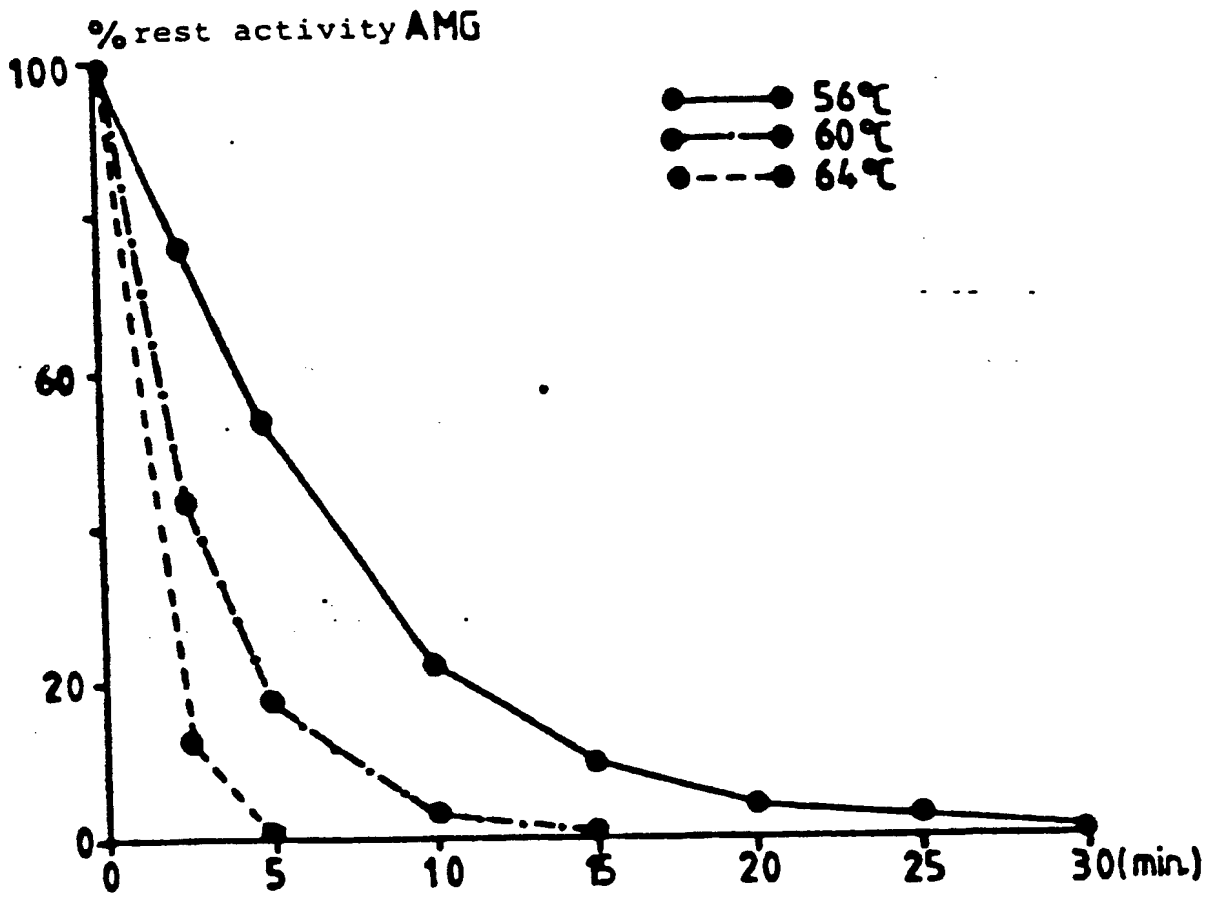


pJDB207 -AMG1



pJDB207-AMG 2

Fig. 16. Inactivation of α-amylase at different temperatures



European Patent Office

EUROPEAN SEARCH REPORT

Application number
EP 86 11 1586

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	WO-A-86 03 778 (BREWING RESEARCH FOUND.) * Pages 1,2; page 4, paragraphs 3-6; pages 13-14: section a; page 17, table II; page 21, section d; page 24, last paragraph; page 26, paragraph 3; claims 4-10 *	1-5,7,8,10,14,19,21-23,25-29,32,36-38,42-55	C 12 N 15/00 C 12 P 21/00 C 12 N 9/30 C 12 N 9/34 C 12 P 7/06 C 12 P 1/00 C 12 C 11/04 C 12 N 1/16 A 21 D 8/04 C 12 N 1/18
Y		12,13,15	

X	EP-A-0 173 668 (SMITHKLINE BECKMAN) * Page 3, lines 9-16; page 5, line 32 - page 6, line 3; page 8, lines 18-23; page 15, lines 9-29; page 19, line 25 - page 22, line 30; claim 30 *	1-4,7,8,14,19,21-23,28,29,32,36-38,42-45,58	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N C 12 P C 12 C
Y		12,13	

X	EP-A-0 163 491 (BIOTECHNICA) * Page 2, line 24 - page 3, line 26; page 4, line 10 - page 5, line 12; page 8, lines 10-13; page 10, lines 15-18; page 13, lines 27-32; claims 2,10,12,15-19 *	1-5,7-10,19,21-23,25-29,32,36-38,42-55	
Y		12,13,15	
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Place of search	Date of completion of the search	Examiner
The Hague	24-04-1987	MADDOX

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
O,X	CHEMICAL ABSTRACTS, vol. 105, no. 7, August 18, 1986, page 168, ref. no. 55627b; Columbus, Ohio, US P.G. MEADEN et al.: "A plasmid vector system for the genetic manipulation of brewing strains." & PROC. CONGR. - EUR. BREW. CONV. 1985, 20th, 219-26. * Abstract *	1-5,7, 8,19, 21-23, 25-28, 32,36, 46-49	

X	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 49, no. 10, October 1985, pages 3089-3091; Tokyo, JP I. YAMASHITA et al.: "Cloning and expression of the Saccharomycopsis fibuligera alpha-amylase gene in Saccharomyces cerevisiae." * Whole article *	1-5,7, 8,14, 16,17, 21-23, 25,26, 28,29, 32,34, 37,38, 40,43-48,54, 55,58	* TECHNICAL FIELDS SEARCHED (Int. Cl.4)

X	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 47, no. 11, November 1983, pages 2689-2692; Tokyo, JP I. YAMASHITA et al.: "Molecular		

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Place of search	Date of completion of the search	Examiner

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
	cloning of a glucoamylase producing gene in the yeast Saccharomyces." * Whole article *	1-5,7, 8,10, 14,19-23,28, 29,36-38,42-45	

X	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 48, no. 7, July 1984, pages 1931-1932; Tokyo, JP I. YAMASHITA et al.: "Secretion of saccharomyces diastaticus glucoamylase from Schizosaccharomyces pombe." * Whole article *	1,5,7, 8,10, 14,19-23,28, 29,36-38,42-45	TECHNICAL FIELDS SEARCHED (Int. Cl.4)

X	PATENT ABSTRACTS OF JAPAN, vol. 10, no. 136 (C-347)(2193), May 20, 1986 & JP-A-60 262 593 (MITSUI TOATSU KAGAKU K.K.) 25-12-1985 * Abstract *	1-5,7, 8,10, 14,19-23,28, 29,36-38,42-45,54	

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	CHEMICAL ABSTRACTS, vol. 105, no. 3, July 1986, page 215, ref.no. 19475e; Columbus, Ohio, US I.S. PRETORIUS et al.: "Molecular cloning and characterization of the STA2 glucoamylase gene of Saccharomyces diastaticus." & MGG, MOL. GEN. HENET. 1986, 203 (1), 29-35. * Abstract *	1-5,7-10,19,21-23,28,29,32,36-38,42	
Y		12,13	

X	FOOD TECHNOLOGY, vol. 38, no. 2, February 1984, pages 99-106,111; Chicago, US C.J. PANCHAL et al.: "Genetic manipulation of brewing and related yeast strains." * Whole article; in particular page 105: "Applications" *	1-8,14,19-23,25-29,32,36-39,42-55	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
Y		12,13,16-18,33-35,40,41,56-58	

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Place of search	Date of completion of the search	Examiner

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	NATURE, vol. 308, no. 5960, April 12, 1984, pages 662-665; Reading, Berks, GB S.J. ROTHSTEIN et al.: "Secretion of a wheat alpha-amylase expressed in yeast." * Page 663, right-hand column *	37,40	
Y		15	

Y	GENE, vol. 25, no. 2/3; November 1983, pages 333-341; Elsevier, Brussels, BE R.J. KLEBE et al.: "A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast." * Page 335, section e *	12,13	
	---		TECHNICAL FIELDS SEARCHED (Int. Cl.4)
Y	CHEMICAL ABSTRACTS, vol. 101, no. 5, July 30, 1984, page 284, ref.no. 35797w; Columbus, Ohio, US C.J. PANCHAL et al.: "Susceptibility of Saccharomyces spp. and Schwanniomyces spp. to the aminoglycoside antibiotic G418." & APPL. ENVIRON. MICROBIOL. 1984, 47(5), 1164-6. * Abstract *	1-58	

X	EP-A-0 125 615 (LABATT) * Pages 1-4,9,10; page 11, paragraphs 2,3; page 12; page 16, last paragraph - page 17, paragraph 1; claims 2-4,8,9,20 *	16-20	
Y		12,13, 33-35 40-58	
	[illegible] ./....		

Place of search	Date of completion of the search	Examiner

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	CHEMICAL ABSTRACTS, vol. 100, no. 9, February 27, 1984, page 312, ref.no. 64776y; Columbus, Ohio, US M.R. DHAWALE et al.: "Interspecific protoplast fusion of Schwanniomyces yeasts." & BIOTECHNOL. LETT. 1983, 5(12), 825-30. * Abstract *	24	

X	BIOTECHNOLOGY, vol. 4, no. 4, April 1986, pages 311-315; New York, US S.A. FILHO et al.: "Stable yeast transformants that secrete functional alpha-amylase encoded by cloned mouse pancreatic cDNA." * Page 314, last paragraph *	37,40	
A		1-36, 38,39, 41-58	TECHNICAL FIELDS SEARCHED (Int. Cl.4)

X	NUCLEIC ACIDS RESEARCH, vol. 14, no. 12, June 1986, pages 4701-4718; IRL Press Ltd, Oxford, GB J.M. PARDO et al.: "Cloning of the STA2 and SGA genes encoding glucoamylases in yeasts and regulation of their expression by the STA10 gene of Saccharomyces cerevisiae." * Abstract *	37,38, 42	

X	EP-A-0 185 327 (SUNTORY) * Page 10, line 29 - page 12, line 8, figure 6a *	37,38, 42,44, 45	

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Place of search	Date of completion of the search	Examiner

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European Patent Office

EUROPEAN SEARCH REPORT

Application number

EP 86 11 1586

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	EP-A-0 096 430 (GIST-BROCADES) * Page 14, lines 3-13 *	1-3,7-9,19,21,22,28,32,36,45	
Y	CHEMICAL ABSTRACTS, vol. 98, no. 11, March 14, 1983, page 135, ref. no. 84345a; Columbus, Ohio, US G. REIPEN et al.: "Nonselective transformation of Saccharomyces cerevisiae." & CURR. GENET. 1982, 6(3), 189-93. * Abstract *	15	
A	BREWERS' GUARDIAN, September 1984, pages 34-37 R.S. TUBB: "Genetic development of yeast strains." * Whole article *	1-58	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
A	EP-A-0 126 206 (CETUS) * Page 3, lines 1-9; page 5, line 32 - page 6, line 3; page 13, lines 18-23, line 31 - page 14, line 18; page 45, lines 15-17; page 46, line 1 - page 47, line 6; claims 21-23 *	1-58	
E	WO-A-86 07 091 (BIOTECHNICA) * Claims 1-5,9-16 *	1-4,7-9,19,21-23,25,27,28,32,36-38,42,44-49,51	

Place of search	Date of completion of the search	Examiner

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